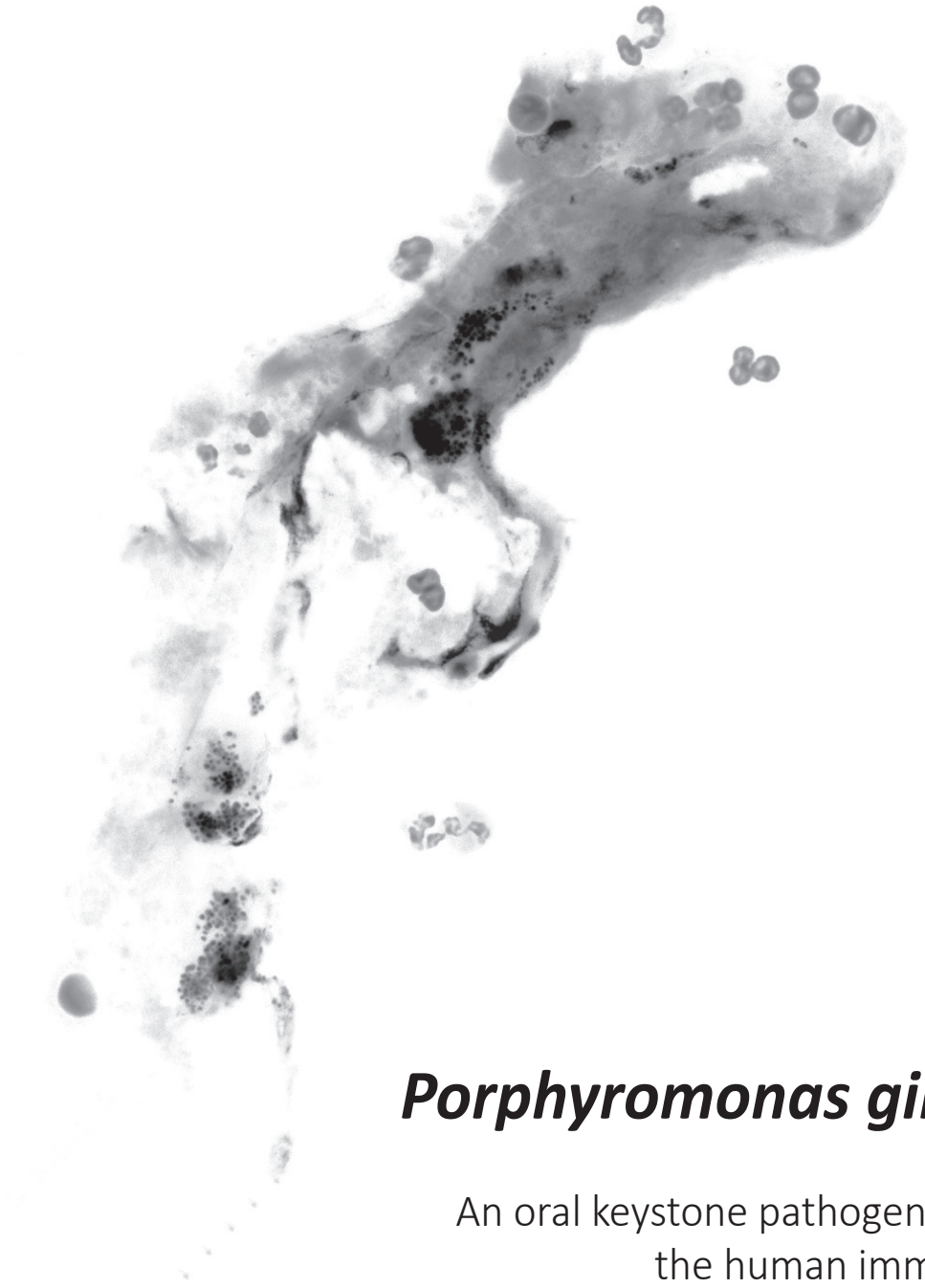

Porphyromonas gingivalis

An oral keystone pathogen challenging the human immune system

Tim Stobernack

The work described in this thesis was performed in the Laboratory of Molecular Bacteriology, Department of Medical Microbiology of the University of Groningen and the University Medical Center Groningen (The Netherlands).

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Porphyromonas gingivalis – An oral keystone pathogen challenging the human immune system

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Table of contents

Supplementary Material is available at http://bit.ly/thesis-supplements

CHAPTER 1

General introduction and scope of this thesis

Periodontitis – a severe inflammation of gum tissue

The clinical background of the research presented in this thesis lies in an inflammatory disease called periodontitis, which affects the tissues surrounding the teeth. Between 10 and 15% of the human population suffers from some type of periodontitis, making it the number one inflammatory disease worldwide[1]. The onset of periodontitis is associated with a combination of genetic factors, as well as environmental factors like inadequate oral hygiene, high bacterial loads and smoking[2]. If left untreated, periodontitis will lead to progressive retraction of the inflamed gingival tissue surrounding the teeth, periodontal bone loss, loosening of the teeth and ultimately tooth loss (Figure 1).

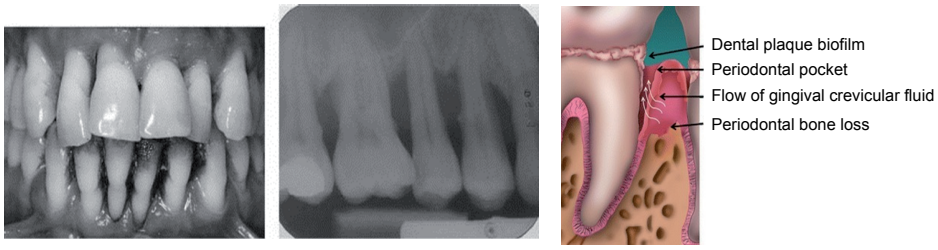


Figure 1: Clinical manifestation of periodontitis. The panels from left to right show a photograph, a radiograph and a schematic representation of the clinical manifestation of a patient with severe periodontitis (images were kindly provided by Arjan Vissink and Johanna Westra).

Gum diseases like periodontitis, or the milder form of it called gingivitis, have existed for thousands of years[3]. In the past, periodontitis was often treated simply by tooth extraction. Nowadays, the treatment ranges from improvement of oral hygiene measures to professional tooth cleaning and periodontal surgery, while tooth extractions are only performed at very advanced stages of the disease. In view of the prevalence of periodontitis and its consequences for human wellbeing, much research has been focused on the triggers and causes of periodontitis, identifying certain bacterial species as potentially causal pathogens for disease development and progression. The species *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* belong to the so-called 'red complex' and, together, they are considered as main causative agents of periodontitis[4]. In addition, the bacterium *Aggregatibacter actinomycetemcomitans* has been implicated in an aggressive form of periodontitis[5,6]. What is often overlooked is the fact that these pathogens are not the only microbes living in our mouth. In fact, more than 600 different bacterial species are known to reside in the human oral cavity along with other micro-organisms, like fungi, amoeba and viruses[7,8]. Importantly, what happens in periodontitis is that the 'eubiotic' homeostasis of microorganisms in the healthy human mouth shifts towards a 'dysbiotic' state, which is characterized by increased abundance of the afore-mentioned red complex pathogens or *A. actinomycetemcomitans*[9]. This dysbiosis can trigger inflammation and damage of the gingival

tissues. As most of the pathogens involved are strict anaerobes, they preferably form biofilms in the periodontal pockets created by swelling of the gingiva and subsequent loss of periodontal attachment of inflamed gingival tissue thereby further promoting the inflammatory state[10,11].

The loss of oral microbial homeostasis and the resulting inflammation lead to a recruitment of innate immune cells towards the infected tissues (Figure 2). Thus, a massive infiltration of neutrophils, and subsequently also macrophages, into the gum tissues can be observed in periodontitis[12,13]. An intensive 'fight' between these immune cells and the bacteria begins. In the course of time, the inflammatory responses, together with highly destructive enzymes produced both by the oral pathogens and host immune cells lead to breakdown of the periodontal tissues, eventually resulting in increased tooth mobility and tooth loss[14]. In recent years, one bacterium in particular became the center of most of the periodontal research, namely *P. gingivalis*, which can be found in more than 75% of all periodontitis patients and which produces a plethora of unique proteins that impact on the human host[15-17].

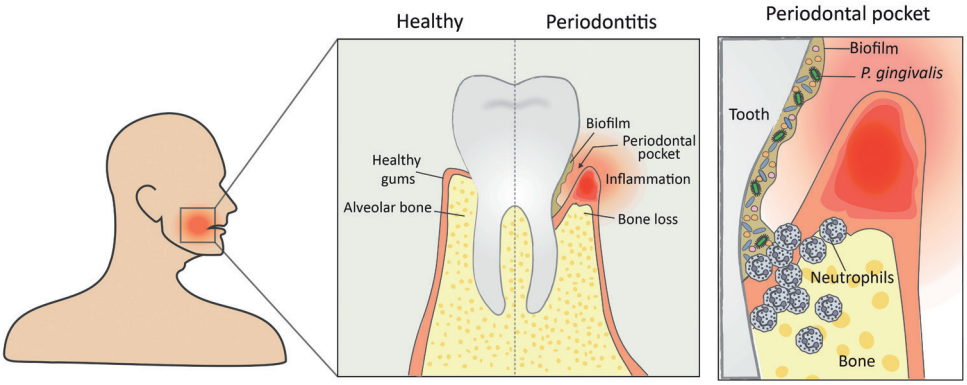


Figure 2: Hallmarks of periodontitis. Schematic representation of biofilm formation and neutrophil recruitment in the periodontal pocket. Note that the periodontal biofilm is polymicrobial, where *P. gingivalis* is represented in green and other microorganisms in orange and blue[10,18].

Porphyromonas gingivalis – the periodontal keystone pathogen

P. gingivalis is a Gram-negative, black-pigmented, non-motile coccoid bacterium, which forms biofilms in the oral cavity (Figure 3)[19]. It is asaccharolytic, which means that it cannot ferment sugars, but needs proteins, peptides and amino acids to thrive. In the oral cavity, *P. gingivalis* maintains its metabolism by a highly proteolytic lifestyle. It produces three different isoforms of cysteine proteases, the so-called arginine-specific gingipains RgpA and RgpB, and the lysine-specific gingipain Kgp[20]. These proteases cleave human proteins, providing small peptides, essential for the bacterial metabolism and growth. Intriguingly, the gingipains are also known to cleave proteins involved in human immune responses, such as immunoglobulins and complement factors, thereby interfering with the host defense and

leading to increased survival of *P. gingivalis*[21-24]. Collectively, the factors leading to increased survival of bacterial pathogens in a host and improved evasion or invasion of immune cells are called virulence factors. *P. gingivalis* avails of a number of these virulence factors[16,25]. Besides the gingipains, *P.* gingivalis can for example produce a strong capsule consisting of polysaccharides[26] that protect *P. gingivalis* from the immune system, and some isolates are highly fimbriated for improved adherence to host tissues[27]. Another important factor is an enzyme called *Porphyromonas* peptidylarginine deiminase (PPAD), which citrullinates arginine residues inside a protein and may protect the bacterium against its own gingipains and allow it to evade the host immune defenses[18].

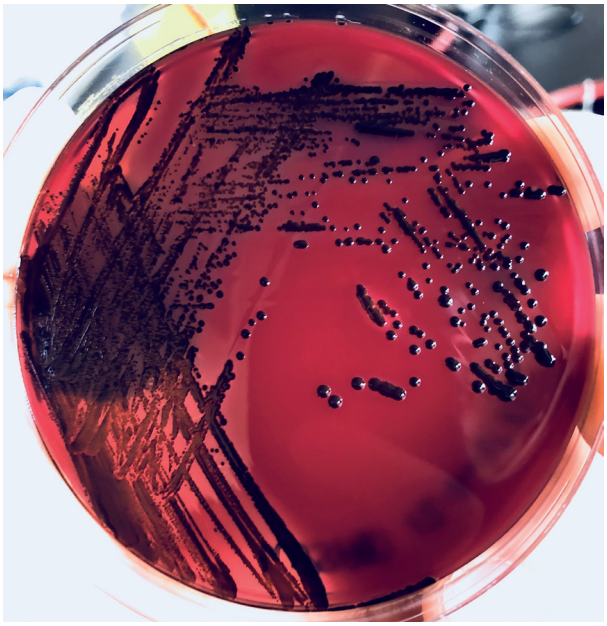


Figure 3: Growth of *P. gingivalis* on a blood agar base No.2 plate for 14 days.

In their struggle with human immune cells, it is crucial for bacterial pathogens to deliver their virulence factors in smart and effective ways. To this end, *P. gingivalis* employs a dedicated secretion system called the type IX secretion system (also: Porin secretion system or PorSS), which targets proteins either towards the outer membrane to which they become attached via an A–lipopolysaccharide (A-LPS) anchor, or secretes proteins directly into the extracellular milieu [28-30]. An alternative way of delivering proteins in the extracellular milieu is the production of outer membrane vesicles (OMVs), which are lipidic vesicles released from the outer membrane[31]. As such, the OMVs represent a distinct compartment for transport of important proteins in a stable manner towards remote destinations. For

example, with the aid of OMVs the bacterium can manipulate immune cells already before getting into close contact with them, and OMVs can function as a decoy for the immune system by binding specific antibodies and thereby protecting the OMV-producing bacterial cell. The main virulence factors of *P. gingivalis*, like PPAD and the gingipains, are secreted by the Porin secretion system, directly as well as by OMV transport (Figure 4) [29,32-34].

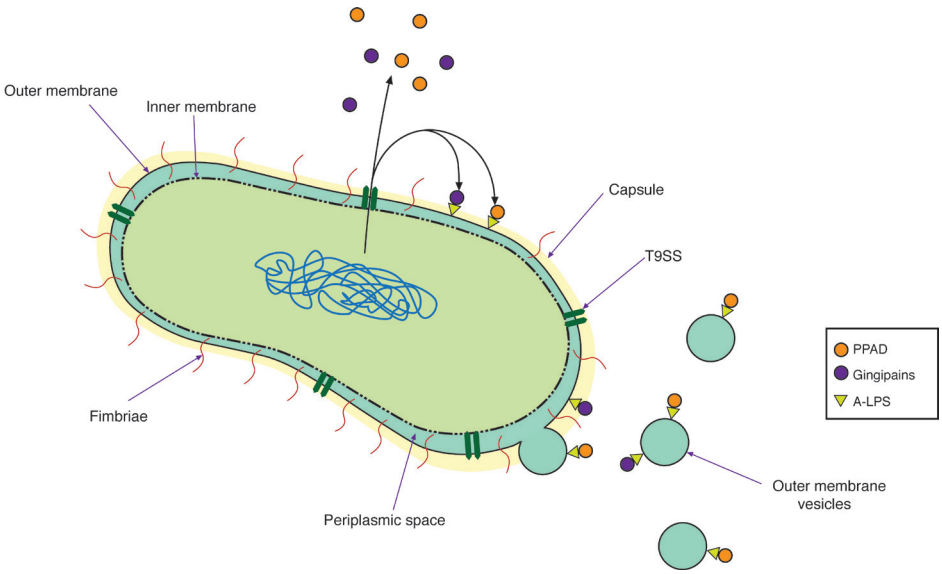


Figure 4: Secretion of virulence factors by *P. gingivalis*. Schematic representation of the secretion and delivery of virulence factors via the type IX secretion system (T9SS) and via production of outer membrane vesicles (OMVs). Upon export from the cytoplasm, PPAD and gingipains either remain attached to the OM or secreted OMVs, or they are secreted in a soluble form into the extracellular milieu. Courtesy of M. du Teil Espina.

Rheumatoid arthritis – a chronic inflammation of the joints

Rheumatoid arthritis (RA) is one of the most common autoimmune disorders in humans. It is characterized by chronic inflammation of synovial joints (Figure 5). The prevalence of RA in the general population is around 0.5-1.0%[35]. However, in patients suffering from periodontitis, the RA prevalence is almost two times as high as in the general population[36-38]. The reason for this might be found in the complex multi-factorial disease pathology of RA. One hallmark and very specific characteristic of RA is the loss of tolerance to citrullinated proteins. Many RA patients develop anti-citrullinated protein antibodies (ACPAs) already years before the actual clinical manifestation of the disease, and they are present in 50% of patients with early rheumatoid arthritis[39]. ACPAs are highly RA-specific auto-antibodies, which are believed to trigger a preclinical state of the disease. Although this causal relationship remains to be

proven unambiguously, ACPAs could lead via several inflammatory cascades to the pathology of chronic inflammation of synovial tissues and damage of articular cartilage and underlying bones.

Just as periodontitis, RA can be triggered by genetic and environmental factors. Human leukocyte antigen (HLA) class II molecules have been associated with a predisposition to RA[40,41]. The disease is more prevalent in the elderly, especially in women, and smoking seems to be another risk factor[42]. As is typical for many multifactorial diseases, it is not entirely clear, what the final trigger for disease onset in the affected individuals is. One theory proposes a so-called two-hit mechanism, where the "first hit" is the formation of autoantibodies like ACPAs or rheumatoid factor (RF)[43,44]. The "second hit" would be delivered by an additional factor, causing a general systemic inflammation, and in combination with the autoantibodies, leading to chronic joint inflammation. One possible "second hit" could be an infection by bacteria, viruses or fungi, or a microbial shift in the gut or the mouth towards a dysbiotic state as mentioned above[11].

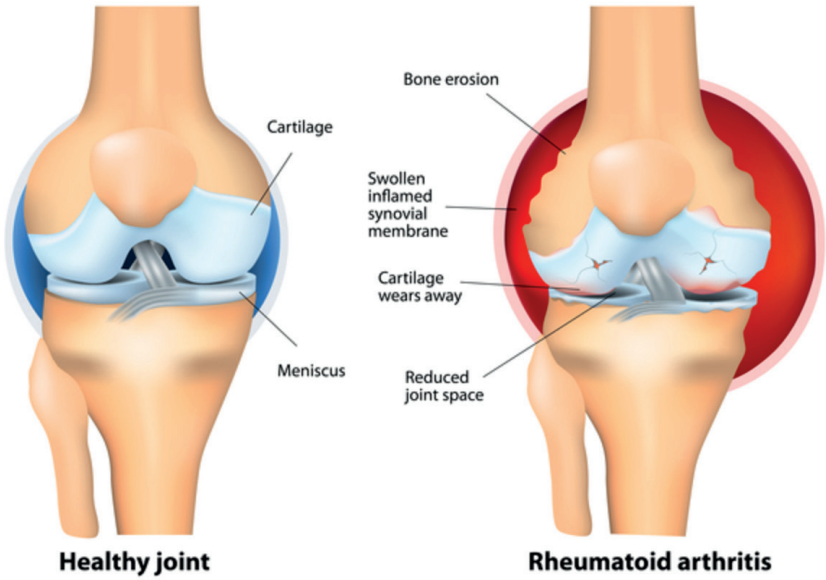


Figure 5: Clinical manifestation of RA (Credit: Designua/Shutterstock.com)

Citrullination and peptidylarginine deiminases – the missing link?

The two-hit hypothesis would be a plausible explanation for the link between periodontitis and RA, but there is also evidence for another mechanistic link between the two diseases. As mentioned above, RA patients lose their tolerance to citrullinated proteins[39]. Citrullination is a post-translational modification of proteins, where positively charged arginine residues in a protein are converted into neutral citrulline residues (Figure 6). It is important in several physiological processes, such as the development of the central nervous system or the keratinization of hair and skin[45,46]. Five different isoforms of human peptidylarginine deiminases (PAD 1-4 and PAD6) can catalyze these reactions. The human PAD enzymes are calcium-dependent and are able to citrullinate any arginine residues inside a protein, irrespective of their internal or terminal location in the polypeptide chain. Intriguingly, *P. gingivalis* produces the aforementioned *Porphyromonas* PAD enzyme (PPAD), which can citrullinate bacterial and human proteins in a calcium-independent manner[47]. In fact, the protein sequence and structure of PPAD is completely different compared to the human PADs, and it has a preference for C-terminal arginine residues[48].

The production of PPAD could be the missing link between the diseases of periodontitis and RA. It has been shown that PPAD is able to citrullinate several known RA auto-antigens, especially the human α-enolase and fibrinogen[49]. By increasing the overall amount of citrullination in periodontitis patients, the burden could become too high and the patients could lose their tolerance at some point. Another possible explanation could be molecular mimicry[50], which relates to the fact that some bacterial proteins are very similar to human proteins (e.g. bacterial vs. human α-enolase). Thus, an immune response against the citrullinated *P. gingivalis* α-enolase could lead to antibodies that cross-react with the citrullinated human α-enolase [51,52]. However, it is not yet entirely clear, what the exact pathways are that lead to the production of ACPAs. In fact, as mentioned above, it is still a matter of debate whether ACPAs are causal agents in RA or are the result of the disease. Nevertheless, ACPAs are strongly associated with RA and are therefore used as a diagnostic marker.

The biological relevance of bacterial citrullination is not entirely unraveled either. Human citrullination clearly is involved in the modification of protein structures and maturation of proteins in developmental processes[46]. Furthermore, citrullination may protect certain proteins against degradation by trypsin-like proteases[53]. So far, the advantage of the PPAD enzyme and citrullination for *P. gingivalis* has not been determined. Several theories for its role have been proposed: *i.* The chemical reaction of citrullination generates ammonia (NH_3; Figure 6) as a byproduct, which has a suppressive effect on neutrophils and could help the bacterium to survive insults by these immune cells [54]. *ii.* PPAD is able to citrullinate and thereby de-activate human host defense proteins[55]. *iii.* PPAD citrullinates other *P. gingivalis* proteins, protecting them from degradation by gingipains and proteases of the host.

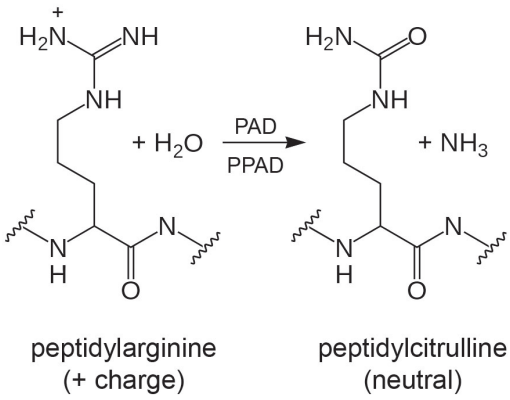


Figure 6: Chemical reaction of citrullination. Peptidylarginine is transformed into peptidylcitrulline by either human PAD enzymes or the bacterial PPAD enzyme. Citrullination changes the overall charge and structure of the respective protein. Ammonia (NH_3) is released as a byproduct.

Proteomics – a powerful tool for 'seeing the bigger picture'

In the last decades, there have been major technological advances in the field of biomedical sciences. With the advent of the so-called 'Omics' approaches, entirely new avenues have been opened for researchers to find answers for their research questions. Since the first whole genome was sequenced in 1995[56], a vast amount of genomics studies was performed in order to unravel the genetic make-up of bacteria, viruses, fungi, humans and other organisms. Such genomics studies give a comprehensive overview of the general make-up of an organism, however without providing information on the actual activation/transcription of the identified genes. Therefore, following the genomics approaches, transcriptomics approaches were developed to provide detailed information about the nature and amounts of all messenger RNAs (mRNA) produced. However, the presence of a gene transcript does not necessarily mean that it is translated into protein. Fortunately, by means of sophisticated mass spectrometry technologies, it is nowadays possible to investigate the whole proteome of an organism. Thus, proteomics can give detailed information on mRNA translation at a global scale, the quantity of individual proteins, post-translational modifications, protein degradation, localization and even activity.

In the bacteriology field, proteomics is nowadays widely applied to achieve a comprehensive understanding of cellular functions and behavior at the systems level. However, it should be noted that the exoproteome, i.e. the extracellular complement of a bacterium, is the main reservoir of virulence factors. The exoproteome was first explored in Gram-positive bacteria, especially *Bacillus subtilis* and *Staphylococcus aureus*, yielding important insights about mechanisms of protein secretion, folding and activity[57-59]. More specifically, major bacterial virulence factors were identified and quantitatively

profiled by mass spectrometry/proteomics approaches[60]. Along the same lines, the proteome of *P. gingivalis* was investigated in several studies[29,32,61]. Nevertheless, a global overview on the *P. gingivalis* proteome and studies investigating the effects of *P. gingivalis* on the human proteome have been scarce to date. Therefore, in most of the studies described in this thesis, proteomics approaches, as illustrated in Figure 7, were applied as a powerful tool to identify features that make *P. gingivalis* a periodontal keystone pathogen, and to define its interactions with human immune cells in periodontitis and RA.

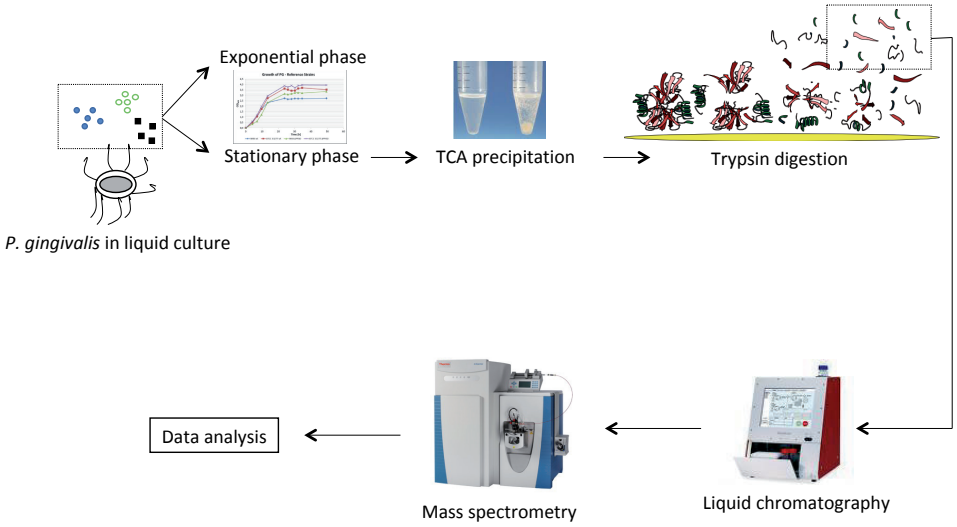


Figure 7: Example scheme of a general proteomics workflow

Scope of this thesis

The main objective of the research described in this thesis was to investigate the interactions of the periodontal keystone pathogen *P. gingivalis* with human innate immune cells. A special focus was placed on the role of bacterial citrullination via the PPAD enzyme and its possible implications in the diseases of periodontitis and RA. As introduced in **chapter 1**, this objective was approached by the application of advanced mass spectrometry. Further, this technology was applied for a detailed comparison of commonly used laboratory strains as well as clinical *P. gingivalis* isolates.

The aim of the study described in **chapter 2** was to investigate the PPAD enzyme of *P. gingivalis* in terms of gene conservation, expression and citrullination ability. The results show that the peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis'.* The PPAD gene was identified in more than one hundred clinical *P. gingivalis* isolates from periodontitis patients, RA patients and healthy control individuals, while it was absent from other related oral bacterial species. Furthermore, the ability of the different clinical isolates for protein citrullination did not differ significantly, leading to the conclusion that the production of PPAD is an invariant trait of *P. gingivalis*, irrespective of the source of isolation.

Chapter 3 of this thesis entitled 'there's no place like OM: vesicular sorting and secretion of PPAD in *Porphyromonas gingivalis'* was aimed at investigating PPAD at the protein level. The results show that, in most of the study isolates, PPAD is mainly present in outer membrane vesicles (OMVs) and to a lesser extent in a soluble state in the extracellular medium. In a small subset of the isolates, the amounts of the OMV-bound PPAD were drastically reduced, and one isolate showed restricted amounts of OMVs. The reduced PPAD binding to OMVs could be associated with a point mutation in the respective gene. It thus seems that such variations have no serious implications for growth and survival of *P. gingivalis* in the oral cavity.

The first two experimental studies described in this thesis focused solely on the PPAD enzyme. In contrast, the study described in **chapter 4** entitled 'extracellular proteome and citrullinome of the oral pathogen *Porphyromonas gingivalis'* gives a global overview on the whole exoproteome of *P. gingivalis.* Several clinical isolates, as well as laboratory strains and PPAD-deficient mutants of *P. gingivalis,* were investigated by mass spectrometry. The isolates displayed a substantial heterogeneity, especially in the presence of typical cytoplasmic proteins in the extracellular fraction. However, the major virulence factors of *P. gingivalis* were shown to be universally expressed at high levels in all investigated isolates. Intriguingly, the arginine-specific gingipain RgpA was found to be citrullinated along with various other extracellular proteins, which has potential implications for periodontitis and RA.

As outlined in Chapter 1, the biological role of the PPAD enzyme for *P. gingivalis* was not fully understood at the start of the present PhD research. **Chapter 5** presents the novel observation that PPAD, 'a secreted bacterial peptidylarginine deiminase, can '*neutralize*' human innate immune defenses'. The research described in this chapter was in particular aimed at unraveling the role of PPAD in the interaction of *P. gingivalis* with the innate immune system. Therefore, neutrophils were infected with PPAD-proficient or PPAD-deficient *P. gingivalis,* and the effects on different aspects of the antimicrobial

activity exerted by neutrophils were examined. The results show that PPAD literally neutralizes human innate immune defenses at three different levels, namely phagocytosis, bacterial capture by neutrophil extracellular traps (NETs) and bacterial killing by a lysozyme-derived antimicrobial peptide. Altogether, this study has shown for the first time that PPAD is a crucial virulence factor of *P. gingivalis* that allows this pathogen to evade the human immune defenses. In fact, PPAD represents a completely new type of immune evasion factor.

The final experimental **chapter 6** of this thesis reports that PPAD, 'a secreted peptidylarginine deiminase of *Porphyromonas gingivalis*, modulates the proteome of human neutrophils and macrophages'. This proteomics study was aimed at capturing the 'bigger picture' of the effects of PPAD on human innate immune cells, neutrophils and macrophages in particular. The results show that PPAD exerts a major influence on the proteome of *P. gingivalis*-infected neutrophils and, to a somewhat lesser extent, the proteome of macrophages. In particular, the abundance of many host defense proteins with antimicrobial activity, histones, oxidative stress-responsive proteins and phagocytosis-related proteins was significantly lower upon infection with the PPAD-proficient bacteria. Importantly, a vast number of proteasome-related proteins, which are involved in the elimination of phagocytosed bacteria, was completely absent from neutrophils infected with PPAD-proficient *P. gingivalis*. Several of these proteins were also found to be citrullinated, suggesting that they may be directly or indirectly connected with the etiology of RA.

Lastly, a 'summary and future perspectives' of the findings described in this thesis are presented in **chapter 7**. In particular, this chapter is focused on the possible implications of *P. gingivalis* in periodontitis and RA. Taking into account that this bacterium is regarded as the keystone oral pathogen, this implies that possible preventive and therapeutic measures to minimize the burden of disease should target the major virulence factors *of P. gingivalis*, especially PPAD and the gingipains.

References

1. Tonetti, M. S., Jepsen, S., Jin, L. & Otomo-Corgel, J. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. *J. Clin. Periodontol.* 44, 456–462 (2017).
2. AlJehani, Y. A. Risk factors of periodontal disease: review of the literature. *Int. J. Dent.* 2014, 182513–9 (2014).
3. Löe, H. Periodontal diseases: a brief historical perspective. *Periodontol. 2000* 2, 7–12 (1993).
4. Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L. Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* 25, 134–144 (1998).
5. Zambon, J. J., Christersson, L. A. & Slots, J. *Actinobacillus actinomycetemcomitans* in human periodontal disease. Prevalence in patient groups and distribution of biotypes and serotypes within families. *J. Periodontol.* 54, 707–711 (1983).
6. Fine, D. H. *et al.* A consortium of *Aggregatibacter actinomycetemcomitans*, *Streptococcus parasanguinis*, and *Filifactor alocis* is present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. *J. Clin. Microbiol.* 51, 2850–2861 (2013).
7. Dewhirst, F. E. *et al.* The human oral microbiome. *J. Bacteriol.* 192, 5002–5017 (2010).
8. Bonner, M. *et al.* Detection of the amoeba *Entamoeba gingivalis* in periodontal pockets. *Parasite* 21, 30 (2014).
9. Hajishengallis, G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* 15, 30–44 (2014).
10. Zijnge, V. *et al.* Oral biofilm architecture on natural teeth. *PLoS ONE* 5, e9321 (2010).
11. Teil Espina, du, M. *et al.* Talk to your gut: the oral-gut microbiome axis and its immunomodulatory role in the etiology of rheumatoid arthritis. *FEMS Microbiol. Rev.* 43, 1–18 (2019).
12. Cortés-Vieyra, R., Rosales, C. & Uribe-Querol, E. Neutrophil Functions in Periodontal Homeostasis. *J. Immunol. Res.* 2016, 1396106–9 (2016).
13. Yang, J. *et al.* Enhanced activity of macrophage M1/M2 phenotypes in periodontitis. *Arch. Oral Biol.* (2017). doi:10.1016/j.archoralbio.2017.03.006
14. Bascones, A. *et al.* Tissue destruction in periodontitis: bacteria or cytokines fault? *Quintessence Int.* 36**,** 299–306 (2005).
15. Griffen, A. L., Becker, M. R., Lyons, S. R., Moeschberger, M. L. & Leys, E. J. Prevalence of *Porphyromonas gingivalis* and periodontal health status. *J. Clin. Microbiol.* 36**,** 3239–42 (1998).
16. Holt, S. C., Kesavalu, L., Walker, S. & Genco, C. A. Virulence factors of *Porphyromonas gingivalis*. *Periodontol. 2000* 20**,** 168–238 (1999).
17. How, K. Y., Song, K. P. & Chan, K. G. *Porphyromonas gingivalis*: An Overview of Periodontopathic Pathogen below the Gum Line. *Front. Microbiol.* 7, 53 (2016).
18. Stobernack, T. *et al.* A Secreted Bacterial Peptidylarginine Deiminase Can Neutralize Human Innate Immune Defenses. *mBio* 9, 456 (2018).
19. Bostanci, N. & Belibasakis, G. N. *Porphyromonas gingivalis*: an invasive and evasive opportunistic oral pathogen. *FEMS Microbiol. Lett.* 333**,** 1–9 (2012).
20. Potempa, J., Sroka, A., Imamura, T. & Travis, J. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: structure, function and assembly of multidomain protein complexes. *Curr. Protein Pept. Sci.* 4, 397–407 (2003).
21. Jung, Y.-J., Jun, H.-K. & Choi, B.-K. Gingipain-dependent augmentation by *Porphyromonas gingivalis* of phagocytosis of *Tannerella forsythia*. *Mol. Oral. Microbiol*. 31, 457–471 (2016).
22. Klarström-Engström, K., Khalaf, H., Kälvegren, H. & Bengtsson, T. The role of *Porphyromonas gingivalis* gingipains in platelet activation and innate immune modulation. *Mol. Oral. Microbiol.* 30, 62–73 (2015).
23. *Porphyromonas gingivalis* Manipulates Complement and TLR Signaling to Uncouple Bacterial Clearance from Inflammation and Promote Dysbiosis. *Cell Host Microbe* 15, 768–778 (2014).
24. Vincents, B. *et al.* Cleavage of IgG1 and IgG3 by gingipain K from *Porphyromonas gingivalis* may compromise host defense in progressive periodontitis. *FASEB J.* 25, 3741–3750 (2011).

25. Lamont, R. J. & Jenkinson, H. F. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol. Mol. Biol. Rev.* 62, 1244–1263 (1998).

26. van Winkelhoff, A. J., Appelmelk, B. J., Kippuw, N. & de Graaff, J. K-antigens in *Porphyromonas gingivalis* are associated with virulence. *Oral Microbiol. Immunol.* 8, 259–265 (1993).

27. Zheng, C., Wu, J. & Xie, H. Differential expression and adherence of *Porphyromonas gingivalis* FimA genotypes. *Mol. Oral. Microbiol.* 26**,** 388–395 (2011).

28. Lasica, A. M., Ksiazek, M., Madej, M. & Potempa, J. The Type IX Secretion System (T9SS): Highlights and Recent Insights into Its Structure and Function. *Front. Cell Infect. Microbiol.* 7, 215 (2017).

29. Sato, K. *et al.* Identification of *Porphyromonas gingivalis* proteins secreted by the Por secretion system. *FEMS Microbiol. Lett.* 338, 68–76 (2013).

30. Gabarrini, G. *et al.* Dropping anchor: attachment of peptidylarginine deiminase via A-LPS to secreted outer membrane vesicles of *Porphyromonas gingivalis*. *Sci. Rep.* 8, 8949 (2018).

31. Schwechheimer, C. & Kuehn, M. J. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat. Rev. Microbiol.* 13, 605–619 (2015).

32. Veith, P. D. *et al. Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J. Proteome Res.* 13, 2420–2432 (2014).

33. Veith, P. D., Luong, C., Tan, K. H., Dashper, S. G. & Reynolds, E. C. Outer Membrane Vesicle Proteome of *Porphyromonas gingivalis* Is Differentially Modulated Relative to the Outer Membrane in Response to Heme Availability. *J. Proteome Res.* 17, 2377–2389 (2018).

34. Gabarrini, G. *et al.* There's no place like OM: Vesicular sorting and secretion of the peptidylarginine deiminase of *Porphyromonas gingivalis*. *Virulence* 9, 456–464 (2018).

35. Silman, A. J. & Pearson, J. E. Epidemiology and genetics of rheumatoid arthritis. *Arthritis Res.* 4 Suppl 3, S265–72 (2002).

36. de Smit, M. *et al.* Periodontitis in established rheumatoid arthritis patients: a cross-sectional clinical, microbiological and serological study. *Arthritis Res. Ther.* 14, R222 (2012).

37. Berthelot, J.-M. & Le Goff, B. Rheumatoid arthritis and periodontal disease. *Jt., Bone, Spine* 77, 537–41 (2010).

38. Detert, J., Pischon, N., Burmester, G. R. & Buttgereit, F. The association between rheumatoid arthritis and periodontal disease. *Arthritis Res. Ther.* 12, 218 (2010).

39. Toes, R. E. & van der Woude, D. ACPA (anti-citrullinated protein antibodies) and rheumatoid arthritis. *Acta Reumatol. Port.* 36, 205–207 (2011).

40. Kampstra, A. S. B. & Toes, R. E. M. HLA class II and rheumatoid arthritis: the bumpy road of revelation. *Immunogenetics* 69, 597–603 (2017).

41. Taneja, V. & David, C. S. Association of MHC and rheumatoid arthritis. Regulatory role of HLA class II molecules in animal models of RA: studies on transgenic/knockout mice. *Arthritis Res.* 2, 205–207 (2000).

42. Stolt, P. *et al.* Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. *Ann. Rheum. Dis*. 62, 835–841 (2003).

43. Payne, J. B., Golub, L. M., Thiele, G. M. & Mikuls, T. R. The Link Between Periodontitis and Rheumatoid Arthritis: A Periodontist's Perspective. *Curr. Oral Health. Rep.* 2, 20–29 (2015).

44. Golub, L. M., Payne, J. B., Reinhardt, R. A. & Nieman, G. Can systemic diseases co-induce (not just exacerbate) periodontitis? A hypothetical 'two-hit' model. *J. Dent. Res* 85, 102–105 (2006).

45. Mohamed, B. M. *et al.* Citrullination of proteins: a common post-translational modification pathway induced by different nanoparticles *in vitro* and *in vivo*. *Nanomedicine* 7, 1181–95–1195 (2012).

46. Baka, Z. *et al.* Citrullination under physiological and pathological conditions. *Jt., Bone, Spine* 79, 431–6 (2012).

47. Gabarrini, G. *et al.* The peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis*. *Sci. Rep.* 5**,** 13936 (2015).

48. Montgomery, A. B. *et al.* Crystal structure of *Porphyromonas gingivalis* peptidylarginine deiminase: implications for autoimmunity in

rheumatoid arthritis. *Ann. Rheum. Dis.* 75, 1255–1261 (2016).

49. Wegner, N. *et al.* Peptidylarginine deiminase from *Porphyromonas gingivalis* citrullinates human fibrinogen and α-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum.* **62,** 2662–72 (2010).

50. Cusick, M. F., Libbey, J. E. & Fujinami, R. S. Molecular mimicry as a mechanism of autoimmune disease. *Clin. Rev. Allergy. Immunol.* 42, 102–111 (2012).

51. Lee, J. Y. *et al.* Association between anti-*Porphyromonas gingivalis* or anti-α-enolase antibody and severity of periodontitis or rheumatoid arthritis (RA) disease activity in RA. *BMC Musculoskelet. Disord.* 16, 190 (2015).

52. Lundberg, K., Wegner, N., Yucel-Lindberg, T. & Venables, P. J. Periodontitis in RA—the citrullinated enolase connection. *Nat. Rev. Rheumatol.* 6, 727–730 (2010).

53. Verheul, M. K. *et al.* Pitfalls in the detection of citrullination and carbamylation. *Autoimmun. Rev.* 17, 136–141 (2018).

54. McGraw, W. T., Potempa, J., Farley, D. & Travis, J. Purification, characterization, and sequence analysis of a potential virulence factor from *Porphyromonas gingivalis*, peptidylarginine deiminase. *Infect. Immun.* 67, 3248–56 (1999).

55. Bielecka, E. *et al.* Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity. *J. Biol. Chem*. 289, 32481–32487 (2014).

56. Fleischmann, R. D. *et al.* Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 269, 496–512 (1995).

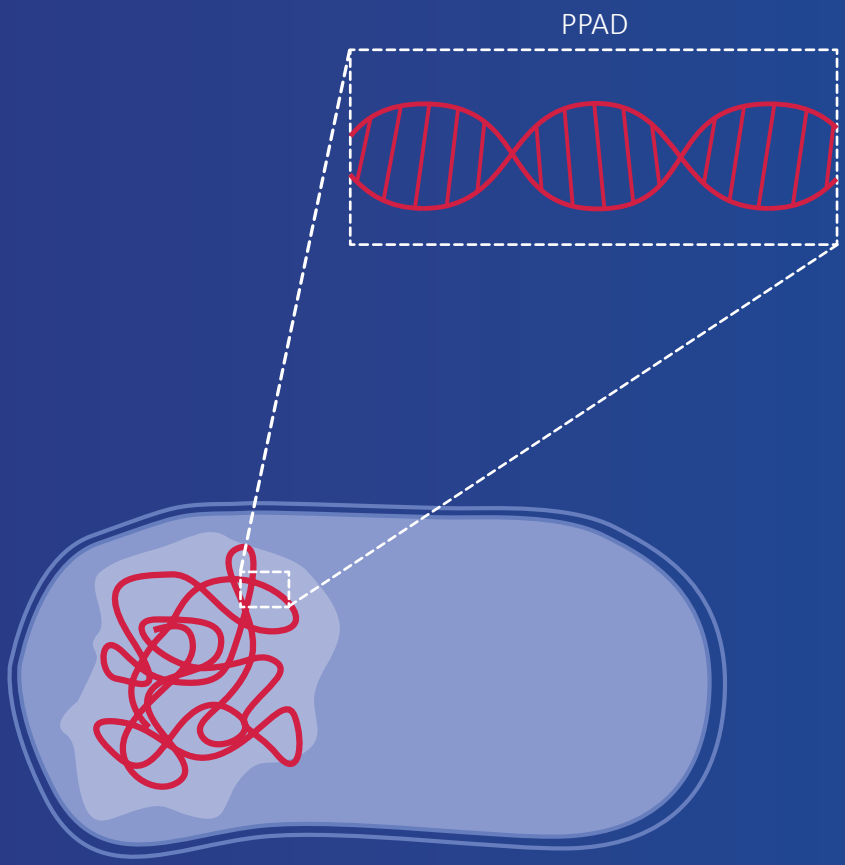
57. Tjalsma, H. *et al.* Proteomics of protein secretion by *Bacillus subtilis*: separating the 'secrets' of the secretome. *Microbiol. Mol. Biol. Rev.* 68, 207–233 (2004).

58. Dreisbach, A., van Dijl, J. M. & Buist, G. The cell surface proteome of *Staphylococcus aureus*. *Proteomics* 11, 3154–3168 (2011).

59. Antelmann, H. *et al.* A proteomic view on genome-based signal peptide predictions. *Genome Res.* 11, 1484–1502 (2001).

60. Sibbald, M. J. J. B. *et al.* Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol. Mol. Biol. Rev.* 70, 755–788 (2006).

61. Lamont, R. J., Meila, M., Xia, Q. & Hackett, M. Mass spectrometry-based proteomics and its application to studies of *Porphyromonas gingivalis* invasion and pathogenicity. *Infect. Disord. Drug Targets* 6, 311–25 (2006).


PPAD

CHAPTER 2

The peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis*

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Abstract

Periodontitis is an infective process that ultimately leads to destruction of the soft and hard tissues that support the teeth (the periodontium). Periodontitis has been proposed as a candidate risk factor for development of the autoimmune disease rheumatoid arthritis (RA). *Porphyromonas gingivalis*, a major periodontal pathogen, is the only known prokaryote expressing a peptidyl arginine deiminase (PAD) enzyme necessary for protein citrullination. Antibodies to citrullinated proteins (anti-citrullinated protein antibodies, ACPA) are highly specific for RA and precede disease onset. Objective of this study was to assess *P. gingivalis* PAD (PPAD) gene expression and citrullination patterns in representative samples of *P. gingivalis* clinical isolates derived from periodontitis patients with and without RA and in related microbes of the *Porphyromonas* genus. Our findings indicate that PPAD is omnipresent in *P. gingivalis*, but absent in related species. No significant differences were found in the composition and expression of the PPAD gene of *P. gingivalis* regardless of the presence of RA or periodontal disease phenotypes. From this study it can be concluded that if *P. gingivalis* plays a role in RA, it is unlikely to originate from a variation in PPAD gene expression.

Introduction

Periodontitis is an infective process that ultimately leads to the destruction of the soft and hard tissues that support the teeth (the periodontium). Periodontitis has been proposed as a candidate risk factor for rheumatoid arthritis (RA) [1]. One of the biologically plausible causal mechanisms accounting for the association between periodontitis and RA could be induction of RA-related autoimmunity at inflamed mucosal sites, e.g. the periodontium[2].

Antibodies against citrullinated proteins (ACPA) are highly specific (98%) for RA[3] and can precede the clinical onset of RA[4]. Citrullination is a post-translational modification catalyzed by a family of enzymes called peptidylarginine deiminases (PAD) [5]. In this reaction, an arginine residue within a protein is converted into the non-coded amino acid citrulline. This modification leads to a loss of positive charge, reduction in hydrogen-bonding ability and subsequently in conformational and functional changes of the protein.

Porphyromonas gingivalis is a major periodontal pathogen involved in destructive periodontal disease[6] and is the only known prokaryote expressing a PAD enzyme[7]. *P. gingivalis* PAD (PPAD) is both a secreted and a cell or membrane vesicle associated enzyme[7]. In contrast to human PAD, PPAD is able to modify free arginine and is not dependent on calcium[7,8]. Citrullination by PPAD enhances the survivability and increases the fitness of *P. gingivalis* due to several immune defense mechanisms. Additionally, a side effect of citrullination is ammonia production, which has a negative effect on neutrophil function and is protective during the acidic cleansing cycles of the mouth[7,8]. PPAD is regarded as a virulence factor because citrullination by PPAD interferes with complement activity[9], inactivates epidermal growth factors[10] and contributes to infection of gingival fibroblasts and induction of the prostaglandin E2 synthesis[11]. Moreover, PPAD has been reported to be able to generate citrullinated forms of various arginine-containing proteins and peptides[8], among which are human fibrinogen and human α -enolase, two candidate auto-antigens in RA[12].

A role of PPAD in autoimmunity is conceivable, considering that citrullinated host peptides generated by *P. gingivalis* are likely to expose epitopes previously hidden to the immune system, which may trigger an immune response in a genetically susceptible host[13]. In fact, cross reactivity has been shown for human antibodies against recombinant CEP-1, an immunodominant epitope of human α -enolase, with *P. gingivalis* enolase[14]. Moreover, there is strong animal experimental evidence supporting the theory that PPAD is the key player linking periodontitis and arthritis[15,16].

Whether expression of PPAD is ubiquitous in *P. gingivalis* and whether there are different forms of the gene among *P. gingivalis* isolates from clinically different donors is currently unknown. Among oral bacteria, citrullination of endogenous proteins has only been shown in the *P. gingivalis* wild-type strain W83 and four clinical isolates from patients with periodontitis without RA12. Related species such as *Porphyromonas endodontalis*, indigenous to the oral cavity, and *Porphyromonas asaccharolytica*, commonly found in the gastrointestinal tract, have not been tested for citrullination capacity.

The aim of this study was to assess expression of the PPAD-encoding gene in representative samples of *P. gingivalis* clinical isolates from patients with and without RA, as well as in related species of

the genus *Porphyromonas* and in the periodontal pathogens *Prevotella intermedia* and *Fusobacterium nucleatum*. Additionally, variation in gene composition was analyzed using a combination of primer sets for the whole gene and for a region including the active site of the gene, by restriction enzyme analysis of the PCR products with three different restriction enzymes, and by whole gene sequencing. Functional analysis of PPAD was carried out by assessment of endogenous citrullination patterns.

Methods

PPAD. *Bacterial strains and culture conditions*. Twelve *P. gingivalis* strains were isolated from 12 consecutive patients with RA and periodontitis, participants of an observational study on periodontitis and RA[17]. Eighty *P. gingivalis* strains were isolated from 80 consecutive subjects without RA (non-RA) with various periodontal diagnoses (periodontitis (n= 75), peri-implantitis (n= 2), gingivitis (n= 1) or a healthy periodontium (healthy carriers, n= 2), recruited for the control group of the same observational study[17]. This study was approved by the Medical Ethics Committee of the University Medical Center Groningen (METc UMCG 2011/010), and conducted in accordance with the guidelines of the Declaration of Helsinki and the institutional regulations. Written informed consent was obtained from all patients. Of note, this study only involved the collection of bacteria; the actual experiments did not involve human subjects and no tissue samples were used. Some general characteristics of the subjects from whom *P. gingivalis* was isolated are listed in Table 1. These clinical isolates, the *P. gingivalis* reference strains ATCC 33277 and W83, *P. asaccharolytica* (clinical isolate), *P. endodontalis* (clinical isolate), *F. nucleatum* (ATCC 25586) and *P. intermedia* (clinical isolate) were anaerobically grown on blood agar plates (Oxoid no. 2, Basingstoke, UK) supplemented with sheep blood (5% v/v), hemin (5 mg/l) and menadione (1 mg/l) and incubated in 80% N_2, 10% H_2 and 10% CO_2, at 37 °C[6].

DNA extraction. Colonies from a blood agar plate were suspended in 500 µL Tris-EDTA buffer and bacterial DNA was isolated utilizing a Precellys 24 Technology tissue homogenizer (Bertin Technologies) (3 times per 30 sec. at 5000 rpm with 30-sec. breaks in between). Afterwards, samples were boiled for 10 min. at 95 °C and centrifuged at 16100 g, 4 °C for 10 min. Supernatants were collected and stored at −20 °C. For whole genome sequencing, total DNA was extracted from 7 *P. gingivalis* strains using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer's instructions.

PPAD PCR. PCR was performed on the PPAD gene using Phusion DNA Polymerase (Thermoscientific) and two sets of primers. The first pair, P1F and P1R, covered the whole gene and the second pair, P2F and P2R, covered a short region around the active site (Cys351). The sequence of P1F was 5′-GGGGAGCTCATGAAAAAGCTTTTACAGGCTAAAGC-3′ while the sequence of P1R was 5′-GGGCTCGAGTTTGAGAATTTTCATTGTCTCACGG-3′. The sequence of P2F was instead 5′-CTGATTCTGAACAACAGGGT-3′, while the sequence of P2R was 5′-TAAAGCTACCGGAACCATTG-3′.

Table 1. General characteristics of subjects from whom *P. gingivalis* was isolated. RA: rheumatoid arthritis, **non-RA:** without rheumatoid arthritis, **IQR**: interquartile range, **n:** number, **DAS28:** disease activity score 28 tender and swollen joint count, **CRP:** C-reactive protein, **anti-CCP**: anti-cyclic citrullinated protein antibody, **IgM-RF:** IgM rheumatoid factor, **MTX:** methotrexate, *for details see[17].

Patient group	number	median age (years, IQR)	current smoker (%)	female (%)
RA	12	64 (56–71)	25	75
non-RA	80	51 (42–60)	27	54
Periodontal diagnosis	**RA (n)**	**non-RA (n)**		
periodontitis	10	75		
peri-implantitis		2		
gingivitis	2	1		
healthy		2		
***Characteristics of RA patients**				
median disease duration (months, IQR)	37 (27–109)			
median DAS28 (IQR)	2.3 (1.6–4.0)			
median CRP (mg/l, IQR)	3 (3–14)			
anti-CCP seropositive (%)	92			
IgM-RF seropositive (%)	92			
MTX monotherapy (%)	92			

The samples were denatured at 98 °C for 10 seconds, annealed at 56 °C for 20 seconds and extended at 72 °C for 2 minutes for a total of 33 cycles. Analysis was then performed using gel electrophoresis on a 1% agarose gel, immersed in SB buffer (10 mM NaOH; 36 mM boric acid, pH 8.0) and subjected to 120 V for 30 minutes.

Restriction enzyme anlysis. DNA samples for restriction enzyme analysis were cleaved with the four-nucleotide cutters *Sau*3AI, *Taq*αI or *Dpn*I following the instructions of the supplier (New England Biolabs) (incubation for 90 minutes at 37 °C for *Sau*3AI and *Dpn*I and at 65 °C for *Taq*αI, followed by heat inactivation for 20 minutes at 80 °C for *Taq*αI and *Dpn*I and at 65 °C for *Sau*3AI). *Sau*3AI and *Taq*α recognize the same sequence (GATC) but cut at different positions while *Dpn*I recognizes TCGA.

Whole genome sequencing and data analysis. DNA was extracted from a representative random sample of 7 *P. gingivalis* isolates that had been obtained from two RA patients with severe periodontitis, two RA patients with moderate periodontitis, two non-RA patients with severe periodontitis and one healthy carrier. The isolates originated from unrelated individuals. The DNA concentration and purity were controlled by a Qubit 2.0 Fluorometer using the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared using the Nextera XT -v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions and then run on a Miseq (Illumina) for generating

paired-end 300 bp reads. *De novo* assembly was performed with CLC Genomes Workbench v7.0.4 (Qiagen, Hilden, Germany) after quality trimming (Qs ≥ 20) with optimal word size[18]. PPAD gene sequences were derived from the 7 assembled genomes and from 5 *P. gingivalis* genomes retrieved from GenBank (accession: NC_002950, NC_010729, NC_015571, CP007756 and AJZS01). DNA and amino acid sequences of 12 PPAD genes were aligned using the MAFFA v7 web server (http://mafft.cbrc.jp/alignment/software/). The PPAD gene sequences of strains 20655, 20658, MDS-16, MDS-45, MDS-56, MDS-85 and MDS-140 have been deposited at DDBJ/EMBL/GenBank under the accession numbers KP862650-KP862656.

Endogenous protein citrullination patters. *Bacterial strains and culture conditions*. The 12 *P. gin- givalis* isolates from patients with RA and 12 randomly selected *P. gingivalis* isolates from non-RA sub- jects, and individual clinical isolates of *P. asaccharolytica*, *P. endodontalis* and *F. nucleatum* were analyzed for endogenous protein citrullination patterns. The isolated bacterial strains were anaerobically grown on blood agar plates (Oxoid no. 2, Basingstoke, UK), which were supplemented with sheep blood (5% v/v), hemin (5 mg/L) and menadione (1 mg/L) and incubated in 80% N_2, 10% H_2 and 10% CO_2, at 37 °C.

Bacterial cell lysate preparation. Four-day old colonies of monocultures of the selected bacterial strains were suspended in sterile phosphate buffered saline (PBS) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics, 1 tablet for 7 ml PBS). After washing and centrifugation cycles (3 × 5 min, 14489 g, 4 °C) the bacterial pellets were resuspended in lysis buffer containing non-denaturing detergent (Noninet P-40, Sigma-Aldrich, Inc.) and sonicated on ice for 15 min. (Bioruptor Standard sonication device, Diagenode s.a.). Protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Pierce Protein Biology Products).

SDS-PAGE and gel stainng. Bacterial cell lysates were prepared with 2× SDS sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 125 mM Tris-HCl (pH 6.8) and 0.02% bromophenol blue) and boiled for 5 min. Per sample, 15 µg of protein was loaded onto a 12.5% SDS-PAGE gel (Criterion Tris-HCl, Bio-Rad Laboratories, Inc.) and resolved by running at 200 V and 15 Watt constant for 1.5 hours. Gels were stained using Coomassie staining (SimplyBlue SafeStain, Life Technologies Corporation) or transferred to a PVDF membrane (Immobilon® EMD Millipore Corporation, Billerica, MA, USA).

Western Blot. Citrulline-containing proteins were detected by Western blotting with a polyclonal IgG antibody (Anti-Citrulline Modified Detection Kit, Upstate, EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions. In addition, detection of citrulline containing proteins was done with a monoclonal IgM antibody (F95) against a deca-citrullinated peptide (U2005- 0033, UAB Research Foundation, Birmingham AL) using the following protocol: after blocking for one hour using a 1:1 dilution of Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, USA) and PBS, incubation with F95 in the same blocking buffer (final dilution 1:2000) with 0.1% Tween 20 (Sigma-Aldrich Co. LLC.) was done overnight at 4 °C. IRDye 800 conjugated goat anti-mouse IgM (Rockland Immunochemicals Inc.,

Gilbertsville) (1:10000) in Odyssey Blocking Buffer and PBS (1:4) with 0.1% Tween 20 (Sigma-Aldrich Co. LLC.) was used as secondary antibody for one hour at room temperature. *In vitro* citrullinated human fibrinogen (341578, Calbiochem, distributed by VWR inter- national) by rabbit PAD (P1584, Sigma-Aldrich Co. LLC.) was used as positive control[19]. Non-specific binding of the secondary antibody was excluded by omitting the primary antibody. Protein bands were detected by the Odyssey system (LI-COR Biosciences, Lincoln, USA). For graphical reproduction of the gels, the signal and size of the protein bands were analyzed using Image Studio Version 2.0.38 (LI-COR Biosciences, Lincoln, USA) with the same image display settings per gel. *F. nucleatum* was considered as negative control[12] and, if present, the signal of the detected bands was corrected for the mean signal of *F. nucleatum*. The sizes of detected bands were plotted in a graph using GraphPad Prism 5 (GraphPad Software Inc.).

Results

PPAD gene is a conserved feature of *P. gingivalis*. The PPAD gene, consisting of 1668 base pairs, was detected by PCR in all 92 investigated *P. gingivalis* strains, but not in any of the other bacterial species tested (Fig. 1A). The same holds true for the region encoding the active site of PPAD, consisting of 328 base pairs (Fig. 1B). Cleavage of the PCR-amplified PPAD genes with three different restriction endo-nucleases and subsequent separation of the fragments by gel electrophoresis revealed no differences in the respective banding patterns for all 92 investigated *P. gingivalis* strains (shown for *Sau*3AI in Fig. 1C). Furthermore, no differences in the whole PPAD gene or in the active site-encoding regions of PPAD were observed between *P. gingivalis* isolates from RA patients or *P. gingivalis* isolates from non-RA patients (shown for the whole PPAD gene in Fig. 1D).

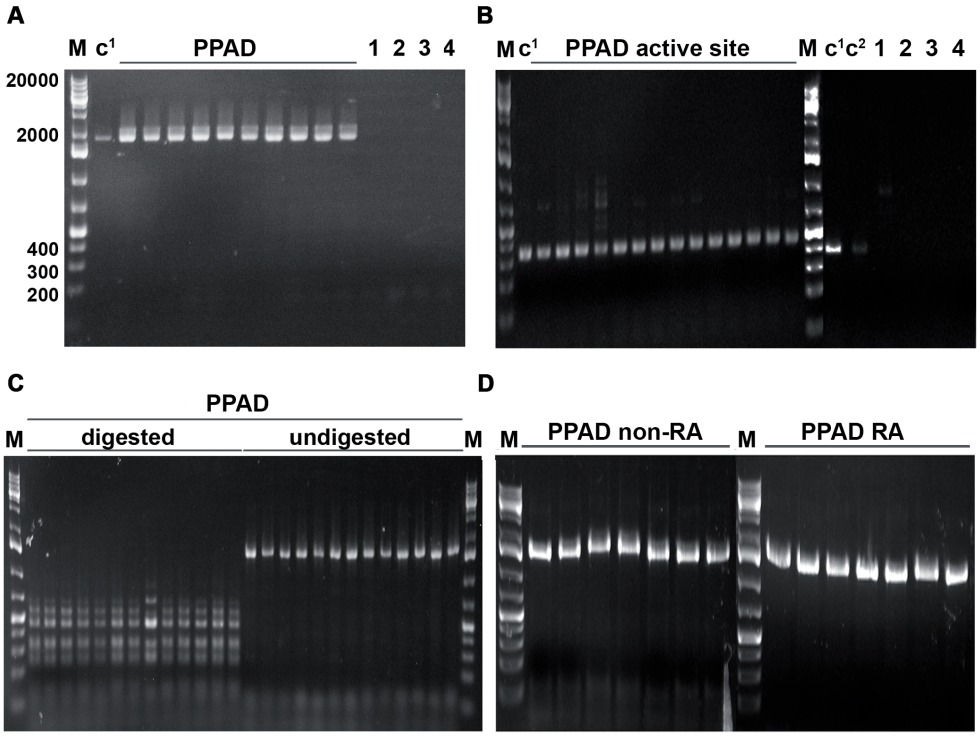

Figure 1. PPAD gene composition analyzed by PCR and restriction enzyme analysis of the PCR products. A. PCR products of PPAD obtained with whole-gene primers (1668 base pairs) using 10 representative *P. gingivalis* isolates of patients without RA. No PPAD genes are detectable in other *Porphyromonas* species or other selected periodontal pathogens. **B.** PCR products of PPAD obtained with active site region primers (328 base pairs) of 14 representative *P. gingivalis* isolates from patients without RA. No PPAD genes are detectable in other *Porphyromonas* species or other selected periodontal pathogens. **C.** Restriction enzyme analysis with *Sau*3AI of PPAD PCR products obtained with whole-gene primers of 13 representative *P. gingivalis* isolates from patients without RA.
D. PPAD PCR products obtained with whole-gene primers of 14 representative *P. gingivalis* isolates from patients with or without RA. **M** = marker displayed as number of base pairs (GeneRuler™ 1 kb Plus DNA ladder). **C¹** = positive control (PPAD of *P. gingivalis* W83). **C²** = positive control (PPAD of *P. gingivalis* ATCC 33277). **1** = *P. intermedia*, **2** = *P. asaccharolytica*, **3** = *P. endodontalis* and **4** = *F. nucleatum.* **Digested** = PPAD PCR products digested with *Sau*3AI. **Undigested** = PPAD PCR products of the same 13 *P. gingivalis* clinical isolates not incubated with *Sau*3AI. **non-RA** = without rheumatoid arthritis. **RA** = with rheumatoid arthritis.

Conservation of PPAD gene sequence. Alignment of the PPAD gene sequences of the *P. gingivalis* strains revealed that the PPAD gene is highly conserved among all analyzed strains. At the DNA and amino acid level, no mutations were found in the signal peptide region and also the active site Cys351 residue is strictly conserved. Overall, the PPAD protein of each strain analyzed has no more than five different amino acids compared to the PPAD proteins of the reference strains W83 or ATCC 33277. In addition, none of the mutations is an insertion, deletion or leads to proteins truncations. However, allelic differences were detected in the PPAD gene sequences, especially for the clinical isolates 20655 (derived from a non-RA patient with periodontitis) and MDS-85 (derived from an RA patient with periodontitis), which displayed 23 and 18 single nucleotide mutations respectively compared to the reference strain W83. Table 2 summarizes the number of nucleotides in the PPAD genes and amino acid residues in the PPAD proteins that differentiate each strain from any other.

Interestingly, the highest number of identified mutations is 25, which separates the PPAD genes from isolates 20655 and 20658 (both derived from non-RA patients with severe periodontitis), and from isolates 20655 (derived from a non-RA patient with severe periodontitis) and MDS-140 (derived from a healthy carrier). Notably, besides their low numbers, the majority of these mutations were synonymous. Taken together, these results show a very high level of PPAD conservation in the investigated *P. gingivalis* isolates.

Table 2. Representation of the numbers of different nucleotides in PPAD genes and numbers of amino acid substitutions in the corresponding PPAD proteins. Top right, number of different nucleotides; bottom left, number of amino acid substitutions (italic). PPAD gene sequences from *P. gingivalis* isolates obtained from two RA patients with severe periodontitis (**MDS-45, MDS-85**), two RA patients with moderate periodontitis (**MDS-16, MDS-56**), two non-RA patients with severe periodontitis (**20655, 20658**) and one healthy carrier (**MDS-140**). Additional PPAD gene sequences were retrieved from GenBank (**W50, HG66, TDC60, W83, ATCC 33277**).

	MDS-45	**MDS-85**	**MDS-16**	**MDS-56**	**20655**	**20658**	**MDS-140**	**W50**	**HG66**	**TDC 60**	**W83**	**ATCC 33277**
MDS-45	0	10	12	9	19	16	14	13	15	11	12	15
MDS-85	*5*	0	14	15	21	18	20	19	19	13	18	19
MDS-16	*3*	*4*	0	11	19	13	12	13	13	11	12	13
MDS-56	*2*	*5*	*3*	0	20	17	11	10	12	12	9	12
20655	*6*	*7*	*7*	*6*	0	25	25	24	22	24	23	22
20658	*3*	*4*	*2*	*3*	*5*	0	18	17	15	15	16	15
MDS-140	*4*	*5*	*3*	*4*	*8*	*3*	0	13	15	17	12	15
W50	*2*	*5*	*3*	*2*	*6*	*3*	*4*	0	12	16	1	12
HG66	*1*	*4*	*2*	*1*	*5*	*2*	*3*	*1*	0	16	11	0
TDC 60	*3*	*6*	*2*	*3*	*7*	*2*	*3*	*3*	*2*	0	15	16
W83	*1*	*4*	*2*	*1*	*5*	*2*	*3*	*1*	*0*	*2*	0	11
ATCC 33277	*1*	*4*	*2*	*1*	*5*	*2*	*3*	*1*	*0*	*2*	*0*	0

Endogenous protein citrullination patterns. To determine possible differenc-re employed. Both assays showed that the patterns of citrullinated proteins of *P. gingivalis* isolates from patients with RA were not detectably different when compared to the pattern of citrullinated proteins from *P. gingivalis* isolates from non-RA patients. Figure 2 (panels A, C, E) shows the Coomassie-stained gel and Western blots for 6 representative *P. gingivalis* isolates of each group, including a graphical representation of the respective citrullination patterns (panels B, D). After correction for conjugate controls, *P. asaccharolytica* and *P. endodontalis* showed no protein bands with the AMC detection method. However, some citrullinated protein bands were observed for these species when the F95 antibody was applied. Neither of the two detection methods revealed citrullinated proteins in samples of *F. nucleatum* (Fig. 2, panels A, C).

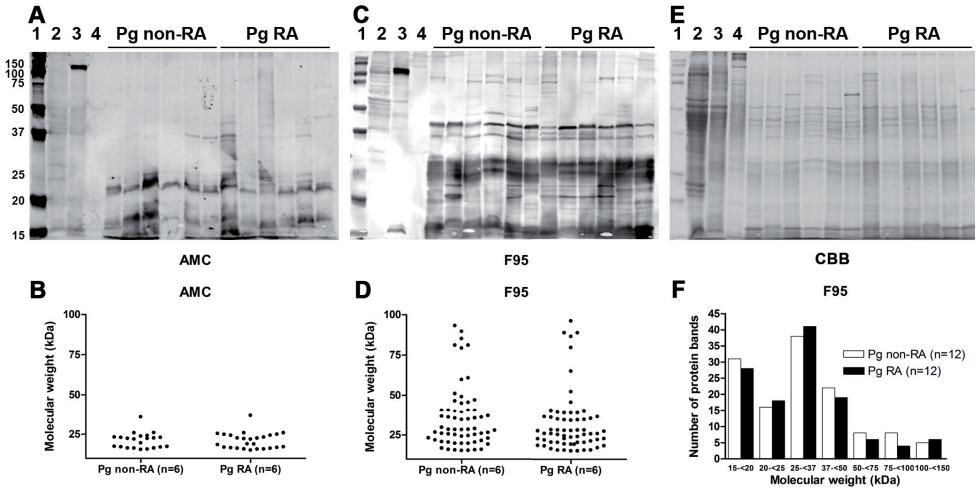

Figure 2. Patterns of citrullinated proteins of *P. gingivalis* isolates from patients with or without RA. (**A,C,E**) Western blots and Coomassie staining of bacterial cell lysates of 12 representative *P. gingivalis* isolates of patients with or without RA (both n= 6). (**A**) Citrullinated protein patterns as detected with the AMC detection method (AMC). (**C**) Citrullinated protein patterns as detected with the F95 anti- citrulline antibody (F95). (**E**) Coomassie staining. (**B, D**) Graphical representation of the Western blots shown in panels (**A, C**). (**B**) Citrullinated protein patterns as detected with the AMC detection method (AMC). (**D**) Citrullinated protein patterns as detected with the F95 anti-citrulline antibody (F95). (**F**) Graphical representation of citrullinated protein patterns as detected by Western blots using the F95 anti-citrulline antibody (F95) against bacterial cell lysates of 24 representative *P. gingivalis* isolates of patients with or without RA (both n= 12). The Western blots were analyzed with the same image display settings. **1** = Molecular weight marker in kilo Dalton (kDa), **2** = *P. asaccharolytica,* **3** = *P. endodontalis,* **4** = *F. nucleatum,* **Pg non-RA** = *P. gingivalis* isolates from subjects without RA, **Pg RA** = *P. gingivalis* isolates of patients with RA. The strong positive staining at circa 120 kDa in *P. endodontalis* (3) both with the AMC and the F95 detection method (panels A, C) is due to non-specific binding of the secondary antibody.

Discussion

This is the first study assessing PPAD expression in a large sample of clinical *P. gingivalis* isolates obtained from patients with or without RA. Our findings indicate that PPAD is omnipresent in *P. gingivalis,* but absent from *P. endodontalis* and *P. asaccharolytica* as well as from the other periodontal pathogens studied.

Our present observations support the view that PPAD may represent one of few, if not the only prokaryotic peptidylarginine deiminase. Of note, our analyses show that the PPAD gene is highly con- served in *P. gingivalis.* Consequently, the encoded PPAD enzymes share 98.9–100% amino acid

sequence identity. This may suggest that PPAD contributes to the ability of *P. gingivalis* to colonize and thrive in its human host. Notably, some mutations in PPAD are missense and it may be of interest to analyze the citrullination levels of the respective PPAD isotypes, in order to see whether these mutations influence the enzymatic activity. Similarly, the mammalian PAD enzyme is also highly conserved with 70–95% identical amino acids sequences[5]; hinting at the importance of protein citrullination for both mammals and *P. gingivalis*, although we found no indications that the PPAD is evolutionarily related to the mammalian PAD enzymes[20].

Concerning PPAD, no differences were noted in the PPAD genes among *P. gingivalis* isolates from patients with or without RA. Also, no differences in PPAD genes were noted among *P. gingivalis* isolates from patients with different stages of periodontal disease or periodontal health. Therefore, we assume that there are no different PPAD variants in *P. gingivalis.* Functional analysis of PPAD further substantiated this assumption. No differences in endogenous citrullination patterns were seen between *P. gingivalis* isolates from RA and non-RA patients, as determined with two different anti-citrulline antibodies. Some differences were observed in the citrullination patterns detected with the two antibodies against citrullinated proteins, which can probably be attributed to the monoclonal (F95) or polyclonal (AMC) nature and the isotypes of these antibodies (IgM and IgG, respectively). Another difference between the anti- bodies is the chemical modification of the citrulline residues in the AMC detection method to ensure detection of citrulline-containing proteins regardless of neighboring amino acid sequences.

Based on the observations in this study, we conclude that PPAD is apparently omnipresent in *P. gingivalis* but absent from *P. asacharolytica* and *P. endodontalis*, two related species of the genus *Porphyromonas.* There are no significant differences in the PPAD gene regardless of RA or periodontal disease phenotypes. Therefore, from this study it can be concluded that if *P. gingivalis* plays a role in RA, it is unlikely to originate from a variation in PPAD gene expression.

An important future goal to strive for will be a detailed characterization of the function of the PPAD protein and its post-translational modifications. The production of recombinant PPAD in *Escherichia coli* has been studied in order to investigate its protein function. The catalytic mechanism was identified and showed different enzyme activities based on an N-terminal truncation of the protein[21]. This finding is in accordance with a recent study by Konig *et al.* which showed that non-cleaved PPAD is autocitrullinated and has decreased activity[22]. Additionally, Konig *et al.* concluded that autocitrullination of PPAD is not the underlying mechanism linking *P. gingivalis* with RA because it does not occur in *P. gingivalis* cells and patient antibodies were directed specifically against non-citrullinated PPAD. Conversely, another recent study showed a peptidyl-citrulline specific antibody response in patients and concluded that PPAD autocitrullination is still a potential mechanism for breaching autoimmunity in RA patients[20]. Besides these theories mainly focusing on cleavage and autocitrullination of PPAD, it will be crucial to investigate the overall citrullination of bacterial and host proteins by PPAD in especially the *in vivo* situation, as well as the interaction of the human PADs with bacterial proteins, as proposed by Quirke *et al.* [20]. In conclusion, it is more likely that a difference in post-translational modification of PPAD might play an important role in RA, rather than a difference in the PPAD gene.

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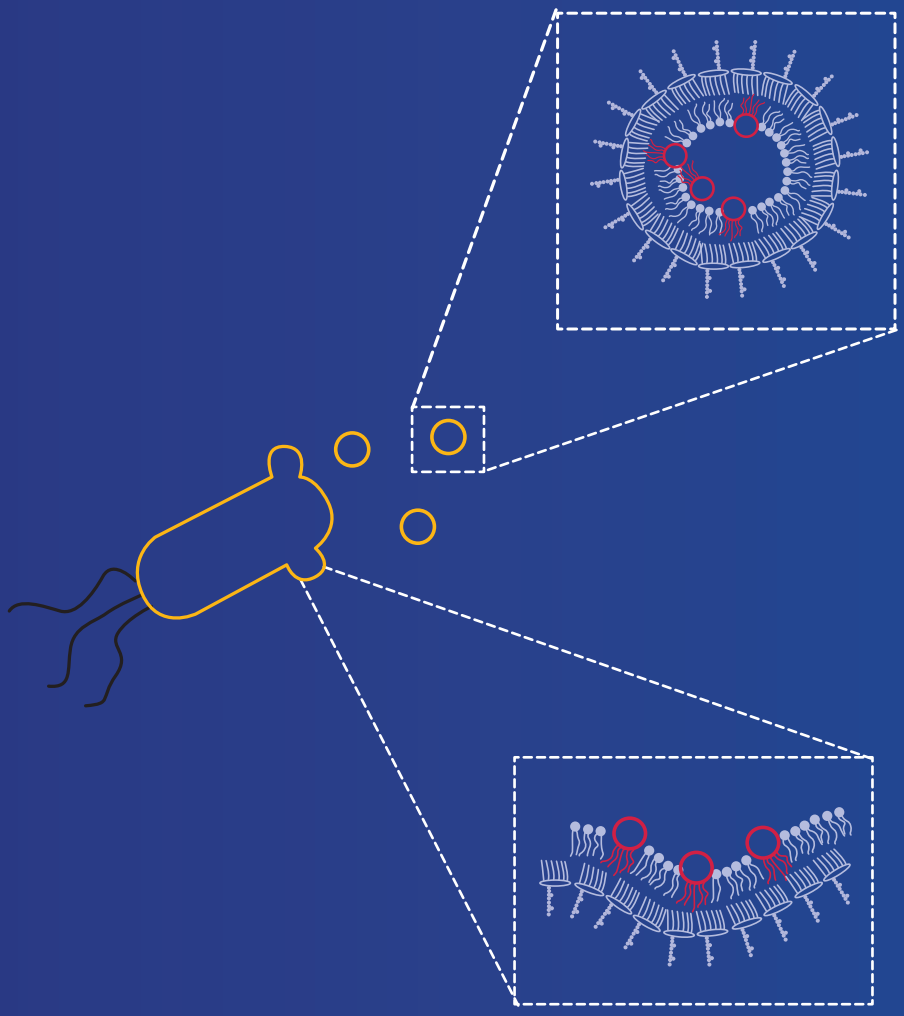
References

1. Chen, H.-H. *et al.* Association between a history of periodontitis and the risk of rheumatoid arthritis: a nationwide, population-based, case-control study. *Ann. Rheum. Dis.* 72, 1206–1211 (2013).
2. Demoruelle, M. K., Deane, K. D. & Holers, V. M. When and where does inflammation begin in rheumatoid arthritis? *Curr Opin Rheumatol* 26, 64–71 (2014).
3. Schellekens, G. A. *et al.* The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum.* 43, 155–163 (2000).
4. Nielen, M. M. J. *et al.* Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum.* 50, 380–386 (2004).
5. Vossenaar, E. R., Zendman, A. J. W., van Venrooij, W. J. & Pruijn, G. J. M. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *Bioessays* 25, 1106–1118 (2003).
6. van Winkelhoff, A. J., Loos, B. G., van der Reijden, W. A. & van der Velden, U. *Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. *J. Clin. Periodontol.* 29, 1023–1028 (2002).
7. McGraw, W. T., Potempa, J., Farley, D. & Travis, J. Purification, characterization, and sequence analysis of a potential virulence factor from *Porphyromonas gingivalis*, peptidylarginine deiminase. *Infect. Immun.* 67, 3248–3256 (1999).
8. Abdullah, S.-N., Farmer, E.-A., Spargo, L., Logan, R. & Gully, N. *Porphyromonas gingivalis* peptidylarginine deiminase substrate specificity. *Anaerobe* 23, 102–108 (2013).
9. Bielecka, E. *et al.* Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity. *J. Biol. Chem.* 289, 32481–32487 (2014).
10. Pyrc, K. *et al.* Inactivation of epidermal growth factor by *Porphyromonas gingivalis* as a potential mechanism for periodontal tissue damage. *Infect. Immun.* 81, 55–64 (2013).
11. Gawron, K. *et al.* Peptidylarginine deiminase from *Porphyromonas gingivalis* contributes to infection of gingival fibroblasts and induction of prostaglandin E2 -signaling pathway. *Mol. Oral. Microbiol.* 29, 321–332 (2014).
12. Wegner, N. *et al.* Peptidylarginine deiminase from *Porphyromonas gingivalis* citrullinates human fibrinogen and α-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum.* 62, 2662–72 (2010).
13. Rosenstein, E. D., Greenwald, R. A., Kushner, L. J. & Weissmann, G. Hypothesis: the humoral immune response to oral bacteria provides a stimulus for the development of rheumatoid arthritis. *Inflammation* 28, 311–318 (2004).
14. Lundberg, K. *et al.* Antibodies to citrullinated alpha-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase. *Arthritis Rheum.* 58, 3009–3019 (2008).
15. Maresz, K. J. *et al. Porphyromonas gingivalis* facilitates the development and progression of destructive arthritis through its unique bacterial peptidylarginine deiminase (PAD). *PLoS Pathog.* 9, e1003627 (2013).
16. Gully, N. *et al. Porphyromonas gingivalis* peptidylarginine deiminase, a key contributor in the pathogenesis of experimental periodontal disease and experimental arthritis. *PLoS ONE* 9, e100838 (2014).
17. de Smit, M. *et al.* Periodontitis in established rheumatoid arthritis patients: a cross-sectional clinical, microbiological and serological study. *Arthritis Res. Ther.* 14, R222 (2012).
18. Zhou, K. *et al.* The mosaic genome structure and phylogeny of Shiga toxin-producing *Escherichia coli* O104:H4 is driven by short-term adaptation. *Clin. Microbiol. Infect.* 21, 468.e7–18 (2015).
19. Vander Cruyssen, B. *et al.* Diagnostic value of anti-human citrullinated fibrinogen ELISA and comparison with four other anti-citrullinated protein assays. *Arthritis Res. Ther.* 8, R122 (2006).
20. Quirke, A.-M. *et al.* Heightened immune response to autocitrullinated *Porphyromonas gingivalis* peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance

in rheumatoid arthritis. *Ann. Rheum. Dis*. 73, 263–269 (2014).

21. Rodríguez, S. B., Stitt, B. L. & Ash, D. E. Expression of peptidylarginine deiminase from *Porphyromonas gingivalis* in Escherichia coli: enzyme purification and characterization. *Arch. Biochem. Biophys.* 488, 14–22 (2009).

22. Konig, M. F., Paracha, A. S., Moni, M., Bingham, C. O. & Andrade, F. Defining the role of *Porphyromonas gingivalis* peptidylarginine deiminase (PPAD) in rheumatoid arthritis through the study of PPAD biology. *Ann. Rheum. Dis.* 74, 2054–61 (2014).



CHAPTER 3

There's no place like OM: vesicular sorting and secretion of the peptidylarginine deiminase of *Porphyromonas gingivalis*

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Abstract

The oral pathogen *Porphyromonas gingivalis* is one of the major periodontal agents and it has been recently hailed as a potential cause of the autoimmune disease rheumatoid arthritis. In particular, the peptidylarginine deiminase enzyme of *P. gingivalis* (PPAD) has been implicated in the citrullination of certain host proteins and the subsequent appearance of antibodies against citrullinated proteins, which might play a role in the etiology of rheumatoid arthritis. The aim of this study was to investigate the extracellular localization of PPAD in a large panel of clinical *P. gingivalis* isolates. Here we show that all isolates produced PPAD. In most cases PPAD was abundantly present in secreted outer membrane vesicles (OMVs) that are massively produced by *P. gingivalis*, and to minor extent in a soluble secreted state. Interestingly, a small subset of clinical isolates showed drastically reduced levels of the OMV-bound PPAD and secreted most of this enzyme in the soluble state. The latter phenotype is strictly associated with a lysine residue at position 373 in PPAD, implicating the more common glutamine residue at this position in PPAD association with OMVs. Further, one isolate displayed severely restricted vesiculation. Together, our findings show for the first time that neither the major association of PPAD with vesicles, nor *P. gingivalis* vesiculation per se, are needed for *P. gingivalis* interactions with the human host.

Key words: PPAD; *Porphyromonas gingivalis*, sorting, secretion, OMVs, OM, RA

Porphyromonas gingivalis is a Gram-negative, anaerobic bacterium and a keystone oral pathogen[1,2]. Albeit mainly studied for its status as causative agent of periodontitis[3], in recent times, newer discoveries have suggested a role for this bacterium in the etiopathogenesis of the autoimmune disease rheumatoid arthritis (RA) [4-9]. RA is a chronic inflammatory disorder that affects the synovium, the tissue enveloping the synovial joints, and if untreated leads to loss of mobility[10-12]. Severe inflammatory responses cause synovial membranes thickening and bone resorption which, in turn, result in deformed joints.

The etiology of rheumatoid arthritis has not been fully comprehended, but it appears that loss of tolerance towards citrullinated proteins plays a significant role[4,8,13,14]. Particularly, autoantibodies against citrullinated host proteins, known as ACPAs (anti-citrullinated protein antibodies), have a remarkable specificity for RA[15,16]. This discovery has shed new light on the link between periodontitis and RA. *P. gingivalis*, in fact, is currently the sole prokaryote reported to secrete an enzyme capable of converting arginine residues to citrulline[4,13,14]. In contrast, humans possess several of such enzymes, collectively called peptidylarginine deiminases (PADs). Remarkably, *P. gingivalis*' PAD (PPAD) is evolutionary unrelated to human PADs. Despite the shared enzymatic activity, PPAD and the human PADs appear to exhibit different substrate specificities. Particularly, PPAD preferentially citrullinates terminal arginine residues of a polypeptide chain, which hints at a relationship with secreted proteases of *P. gingivalis*, the so-called gingipains RgpA and RgpB in particular. These gingipains cleave proteins at arginine residues, thereby creating a perfect target for PPAD. Notably, PPAD was previously shown to be present in two variants, an outer membrane (OM)-bound state and a soluble secreted state[17,18]. This distinct feature of PPAD appears directly related to the transport system responsible for its export, the Por secretion system[17,19]. During export, a fraction of the PPAD is attached to the OM *via* A-LPS anchoring, which involves cleavage of the C-terminal Por-specific signal peptide by the putative sortase PorU[17,20-22]. Moreover, PPAD was proposed to reside also in outer mem- brane vesicles (OMVs). These secreted nanostructures result from a specific OM blebbing process that, in the case of *P. gingivalis*, is not yet fully understood[2]. OMVs are generally produced as single bilayer membranous structures for various functions, such as shuttling their cargo of proteins to the outside of the cell or delivering it to targets in the extracellular milieu[23]. For *P. gingivalis*, the OMVs were suggested to have a role in pathogenesis, considering that their cargo appears to be mainly composed of virulence factors[2,23,24].

Thus far, it was not known whether clinical *P. gingivalis* isolates invariably express and secrete PPAD. The aim of this study was therefore to investigate the extracellular localization of PPAD in a large panel of clinical *P. gingivalis* isolates. This was first tested by Western blotting using unfiltered growth medium fractions of 93 clinical isolates and two type strains. In principle, such growth medium fractions contain both soluble secreted proteins and OMV-associated proteins. Indeed, PPAD was detectable in the growth media of all isolates, and the PPAD signal was absent from samples of two genetically engineered PPAD deletion mutants (Figs. 1 and S1). Unexpectedly, two classes of isolates (hereafter referred to as PPAD "sorting types") were distinguished based on different PPAD banding patterns. The first, most common, sorting type I produces a major PPAD species of 75–85-kDa, running as a broad band on lithium dodecyl sulfate (LDS)-PAGE, *plus* a minor PPAD species of 47-kDa. Some type I isolates also produce a third PPAD species of 60-kDa (Figs. 1 and S1). The PPAD sorting type II, rep- resented

by only 9 isolates, displays massively reduced levels of 75–85-kDa species. Further, the type II isolates produce the 47-kDa species *plus* a PPAD species of 37-kDa. Some also produce relatively small amounts of the aforementioned 60-kDa species.

To verify whether any of the secreted PPAD species are also present in cells of *P. gingivalis*, we analyzed cells of *P. gingivalis* isolates belonging to either PPAD sorting type by Western blotting (S2 Fig.). Cells of the type I isolates, displayed only the 75–85-kDa species. In contrast, cells of the sorting type II isolates (513324 and 513044) displayed only the 37-kDa PPAD species. Of note, cells of both sorting types lack the 47-kDa PPAD species detected in growth medium fractions, showing that this species represents a soluble secreted form of PPAD. These findings are fully consistent with the previous reports by Konig *et al.* [25] and Shoji *et al.* [26], who proposed that the 75– 85-kDa species represents the A-LPS-modified OM-bound form of PPAD, while the 47-kDa species represents a soluble secreted form of PPAD. The A-LPS modification would explain the thick banding pattern displayed by the 75–85-kDa PPAD species upon LDS-PAGE (Fig. 1).

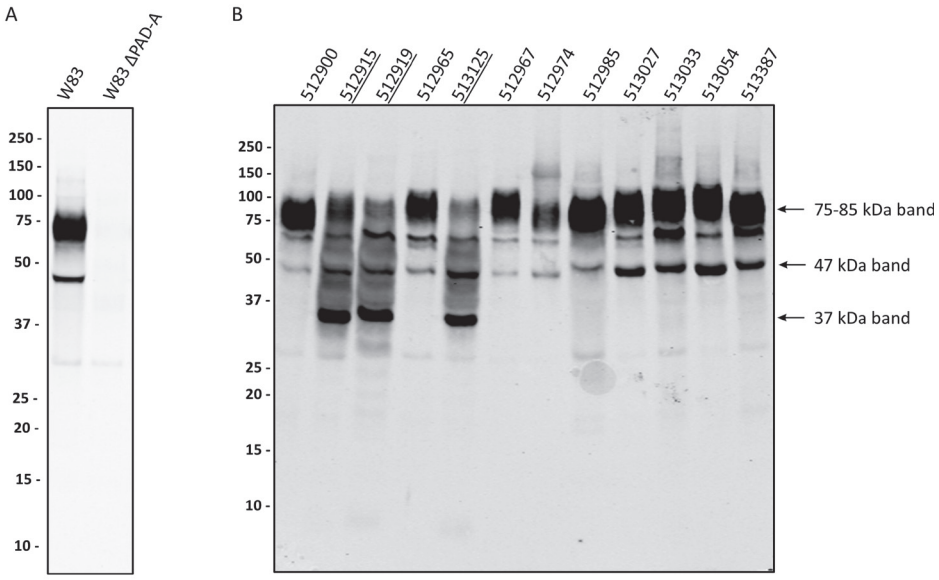

Figure 1: Distinction of PPAD sorting types I and II. *P. gingivalis* isolates were cultured for four days in BHI medium. Subsequently, bacterial cells were separated from the growth medium, and growth medium fractions were used for immunoblotting with PPAD-specific antibodies. (A) *P. gingivalis* reference strain W83 and the isogenic PPAD deletion mutant. (B) *P. gingivalis* clinical isolates. Names of sorting type II isolates are underlined. Molecular weights of marker proteins and different PPAD species are indicated.

Previous analyses have shown that *P. gingivalis* secretes OMVs[2,23-25,27]. It is thus conceivable that the secreted 75–85-kDa A-LPS-modified PPAD species is associated with OMVs. To test this idea, we analyzed OMVs collected from spent growth medium fractions by ultracentrifugation for the presence of PPAD. Indeed, the 75–85-kDa species of type I and II isolates was pelleted with the OMVs and no longer detectable in the supernatant after ultracentrifugation (Fig. 2). Consistent with the literature data, the 47-kDa species of PPAD fractionated with the ultracentrifugation supernatant showing that this is a soluble secreted form of PPAD. Notably, the 37-kDa PPAD species displayed a dual localization, being detectable both in the OMV and supernatant fractions (Fig. 2). This OMV association of the 75–85-kDa and 37-kDa PPAD species is consistent with the detection of these species in *P. gingivalis* cells (S2 Fig.).

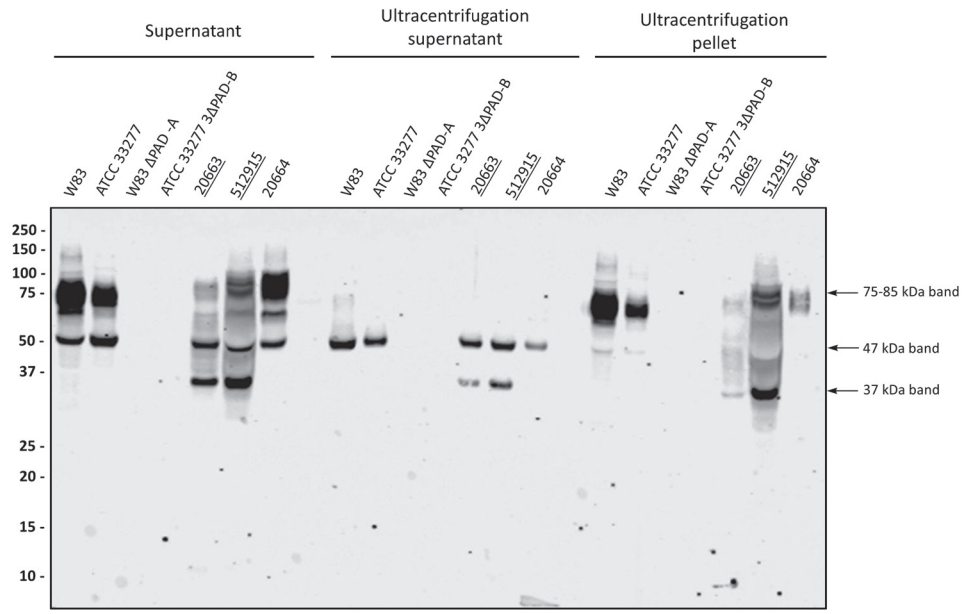

Figure 2: Association of PPAD species with OMVs. Growth medium fractions (designated 'supernatant') of *P. gingivalis* sorting type I and II isolates were subject to ultracentrifugation. Subsequently, the supernatant and pellet fractions were analyzed by immunoblotting as indicated for Figure 1. Samples relating to the reference strain W83 and ATCC 33277, the respective PPAD deletion mutants, and sorting type I and II isolates are indicated with names of type II isolates underlined. Molecular weights of marker proteins and different PPAD species are indicated.

Considering that the presence of the 75–85-kDa species in the medium is associated with OMVs, it was conceivable that the sorting type II might relate to impaired production of OMVs. This was investigated by inspecting the possible presence of OMVs in ultracentrifuged cell-free growth medium fractions of three type II isolates using transmission electron microscopy (TEM). OMVs were observed in

all three samples, as was the case for the type strain W83 and a clinical isolate, both belonging to sorting type I (Fig. 3). Of note, the sorting type II isolate MDS33 displayed very low amounts of OMVs, showing that massive vesiculation, as displayed by all other investigated clinical *P. gingivalis* isolates, is not an essential trait for survival of this pathogen in the human gingiva. Furthermore, observed variations in the vesiculation levels and OMV shapes (Fig. 3) did not correlate with the sorting types of the respective strains.

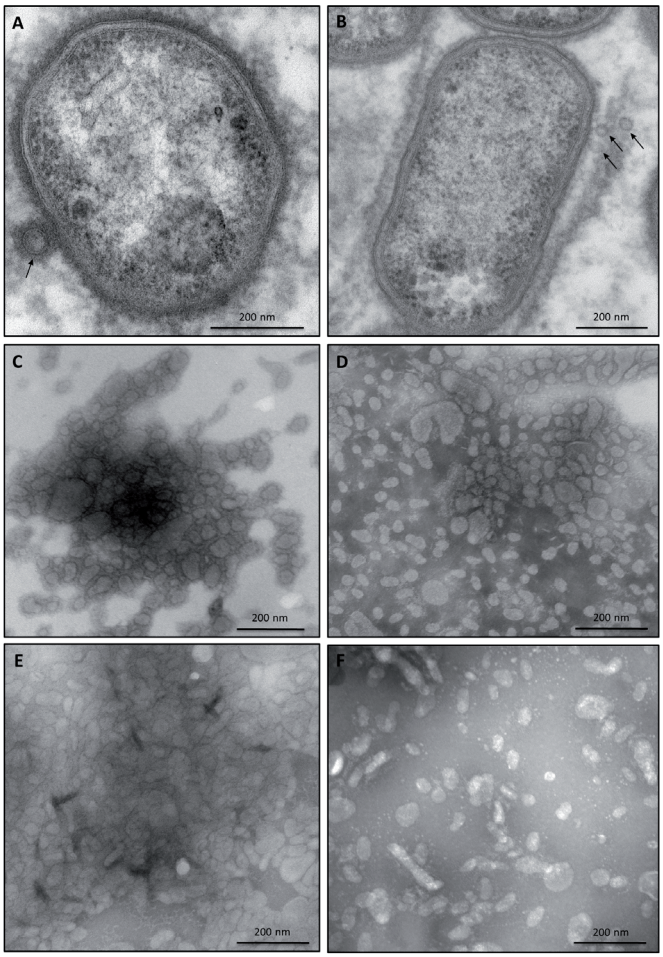

Figure 3: OMV formation by sorting type I and II isolates of *P. gingivalis*. Electron micrographs of vesiculating cells of (A) the *P. gingivalis* type strain W83, and (B) the sorting type II isolate MDS33. Electron micrographs of OMVs collected from (C) strain W83, (D) the sorting type I isolate 505700, and the sorting type II isolates (E) 505759 and (F) 512915.

The strikingly different PPAD banding patterns of the two sorting types prompted further analyses to verify whether other secreted proteins might display similar distinctive features. As shown by Western blotting, the extracellular appearance of the OM protein Omp41 and the secreted gingipains Kgp, RgpA and RgpB did not follow the classification of the PPAD sorting types (S3 and S4 Figs.). Notably, the isolate MDS33 displayed low levels of vesiculation but, for unknown reasons, the levels of Omp41 secreted into the growth medium were still com- parable with other isolates. Together, these observations imply that the type I and II sorting types represent a specific shared feature of the respective PPAD proteins.

P. gingivalis is renowned for its high proteolytic activity, partially due to its secreted gingipains. As shown by Western blotting, the two sorting types did not display major differences in gingipain secretion (S4 Fig.). To exclude the possibility that the suppressed appearance of the 75–85-kDa PPAD species in the type II isolates is due to proteolytic activity, type II isolates were grown in the presence of EDTA-free or regular protease inhibitors. Intriguingly, this resulted in decreased levels of the secreted 75–85-kDa species, relative to the 47-kDa and 37-kDa species (Figs. 4 and S5), suggesting a reduced export of PPAD or a reduced level of post-translational modification rather than reduced proteolysis. Together, these observations imply that the suppressed appearance of the 75–85-kDa species in type II isolates is probably not due to gingipain activity. Conversely, the possible involvement of proteases that are not inhibited by standard protease inhibitors in a conversion of the 75–85-kDa species to, for example, the 37-kDa species cannot be excluded.

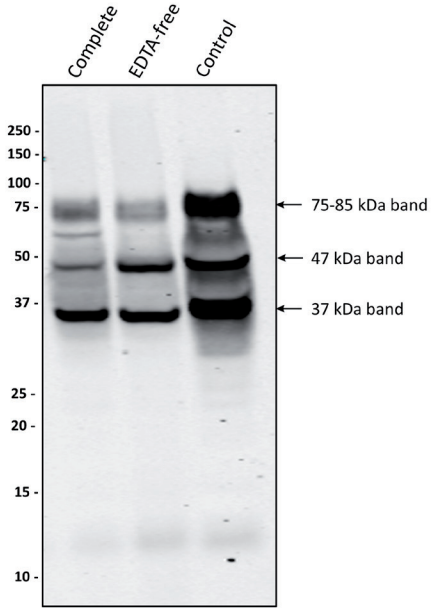

Figure 4: Protease inhibitors suppress the formation of a 75-85-kDa PPAD species. *P. gingivalis* sorting type II isolate 20663 was grown in the presence or absence of protease inhibitors as indicated. Growth medium fractions were analyzed by immunoblotting as indicated for Figure 1. Molecular weights of marker proteins and different PPAD species are indicated.

Since the PPAD sorting type is apparently not related to proteolysis or strain-specific differences in OMV formation, we investigated whether particular residues in PPAD can be associated with sorting type I or II. To this end, the nine sorting type II isolates were sequenced and the encoded PPAD proteins were compared to those of previously sequenced type I isolates[28]. This showed a high degree of PPAD conservation (S5 Table) [28]. Residues involved in catalysis (Asp130, His236, Asp238, Asn297 and Cys351) or substrate specificity (Arg152 and Arg154) [29] are invariably present. Compared to the type I strain W83, the highest numbers of PPAD amino acid substitutions are observed in the type II isolates MDS33 and 512919 (S5 Table). However, only the Q373K substitution is distinctive for all identified type II isolates. This implies that Gln373 is needed to produce the dominant 75–85-kDa PPAD species associated with OMVs. Notably, 3D modelling maps Gln373 to the outer surface of the PPAD protein (Fig. 5A), where this residue's side-chain has the potential to interact with other molecules or OMVs. Interestingly, the Q373K substitution alters the electrostatic surface potential of the protein (Fig. 5B and C). Of note, several substitutions observed for type II isolates map to positions that allow interactions with the outer environment. In particular, substitutions at positions 77, 153, 203, 231, 232, 235, 291 and 335 map to the surface of PPAD (Fig. 5D). In fact, these substitutions are located in the vicinity of the major catalytic residue Cys351, suggesting possible alterations in the catalytic activity or substrate specificity of the respective PPAD molecules (Fig. 5D). This is surmised by the massive variations in electrostatic surface potential high-lighted through 3D modeling (Fig. 5E and F).

In conclusion, PPAD is expressed by all 93 investigated *P. gingivalis* isolates, demonstrating that this is a highly conserved feature of *P. gingivalis*[28]. Interestingly, we identified two different PPAD sorting types based on the PPAD banding patterns upon LDS-PAGE. Both sorting types produce the 47-kDa soluble secreted form of PPAD. However, the 82 type I isolates produce high levels of a presumably A-LPS-modified 75–85-kDa OMV-associated PPAD species, which is detectable only in very low amounts in the type II isolates. Conversely, the type II isolates produce a 37-kDa form of PPAD that is detectable both in OMVs and in the OMV supernatant after ultracentrifugation. At present, the origin of the 37-kDa species is enigmatic, but it could be related to proteolysis during its export from the cytoplasm. If so, this cleavage is not affected by inhibitors that inactivate the most common classes of proteolytic enzymes, although it is difficult to exclude the possibility that the activity of some proteases was not or only partially inhibited under the present experimental conditions. Importantly, appearance of the 37-kDa species in type II isolates cannot be correlated to the production of gingipains, especially since gingipains are abundantly produced by all type I and II isolates. On the other hand, the levels of the 75–85-kDa PPAD species are associated with a Q373K substitution, where Gln373 is diagnostic for the type I sorting phenotype and Lys373 for the type II phenotype. As the 75–85-kDa species is allegedly A-LPS-modified, our findings implicate Gln373 in this particular modification. In this respect, it is noteworthy that A-LPS modification occurs upon C-terminal cleavage by the sortase-like transpeptidase PorU[17,20-22]. To date, the precise mechanism involved in A-LPS modification is not entirely clear, but the available data suggest that PorU replaces the Por-specific C-terminal signal peptide with an A-LPS-related modification through transpeptidation at a Ser residue[22]. In turn, this implies that the surface-

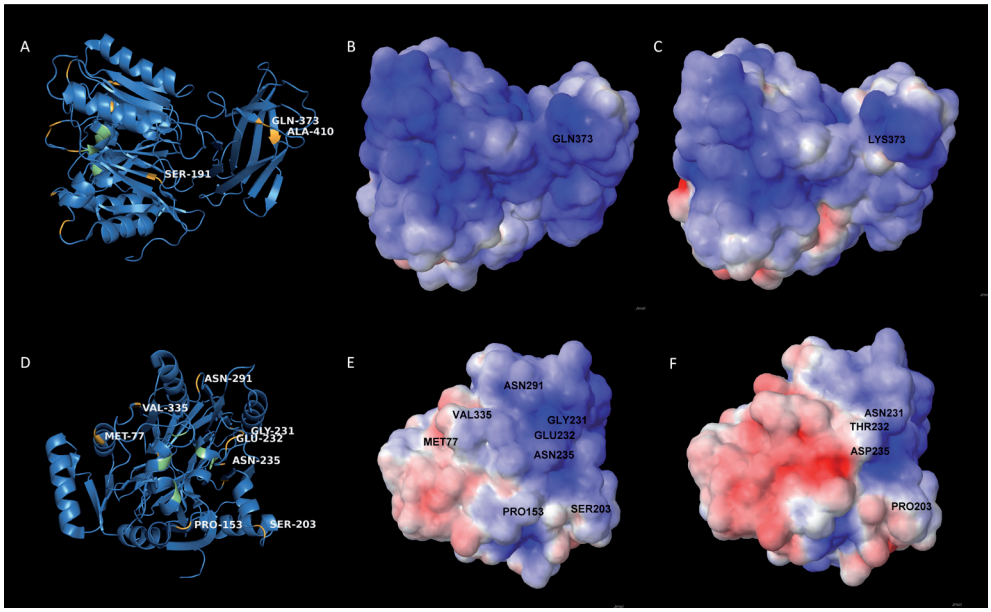

Figure 5: Position of amino acid substitutions in PPAD and their impact on the electrostatic potential of the protein. (A) 3D-structural ribbon representation of the PPAD protein from *P. gingivalis* reference strain W83, showing in yellow surface-exposed amino acid residues that have been substituted in PPAD proteins from other *P. gingivalis* sorting type II isolates. Catalytic residues of PPAD are shown in green. (B and C) Electrostatic potential maps showing, respectively, the PPAD proteins of strain W83 and the sorting type II isolate MDS33 from the same perspective. The two maps display the difference in electrostatic potential (red represents -5 *KT/e* and blue +5 *KT/e*), and the respective Gln or Lys residues at position 373 are indicated. (D) 3D-structural ribbon representation of the PPAD protein from *P. gingivalis* strain W83, showing surface-exposed amino acid residues that have been substituted in other PPAD proteins (marked in yellow) of sorting type II isolates from the perspective of the catalytic site. The catalytic residues are marked in green. (E) Electrostatic potential map of the PPAD protein of strain W83, displaying all the residues subject to substitutions in sorting type II isolates. (F) Electrostatic potential map of the PPAD protein from the sorting type II isolate MDS33.

exposed Gln373 residue is probably not a target for PorU-mediated A-LPS modification. Indeed, in a previous study we have shown the presence of unmodified Gln373 containing peptides by mass spectrometry, although these may have been derived from the 47-kDa PPAD species[30]. Moreover, the detection of minor amounts of the 75–85-kDa species in type II isolates suggest that A-LPS modification is not fully impaired when PPAD bears the Q373K substitution. Altogether, these observations imply that Lys373 interferes with the A-LPS modification of PPAD, possibly through the altered surface charge as the lysine side chain is positively charged while that of glutamine is neutral. Although this is an attractive idea, further studies combining site-specific mutagenesis of PPAD and subcellular localization experiments are needed to define the precise roles of Gln373 and Lys373 in the sorting of PPAD.

Lastly, the level of OMV-bound PPAD does not appear to directly correlate with a diagnosis of RA in the host. Yet, it has to be noted that RA is a multi-factorial disease and, as such, the PPAD sorting type of a *P. gingivalis* isolate might still be a factor that contributes to the overall citrullination burden. In particular, OMVs are generally considered to serve as delivery vehicles for virulence factors that are readily internalized by phagocytic cells, especially neutrophils and macrophages. In turn, this could lead to the presentation of citrullinated peptides, leading to the formation of ACPAs. In this case, sorting type II *P. gingivalis* isolates would have a lowered propensity for generating ACPAs. This is an intriguing hypothesis that merits further research, because it would mean that the bacterial machinery for A-LPS modification of PPAD could be a druggable target for the fight against RA.

Materials and Methods

Bacterial strains and culture conditions. 90 *P. gingivalis* isolates (S1 Table) were collected in Groningen, the Netherlands, from patients with a periodontal diagnosis, of which eight were confirmed to have RA. Additionally, the study isolates included one *P. gingivalis* isolate from a healthy carrier, and two *P. gingivalis* type strains (W83 and ATCC 33277), with the respective engineered PPAD deletion mutants[31]. As controls, two unrelated oral pathogens, *Prevotella intermedia* and *Fusobacterium nucleatum*, were included. Each isolate was grown anaerobically as previously described[30], the cultures being inoculated with 4 days old colonies on blood agar plates.

Escherichia coli BL21 was cultured aerobically in Lysogeny Broth (LB) at 37°C and with shaking (250 rpm), or on LB agar at 37oC. *Lactococcus lactis* was grown without shaking in M17 broth supplemented with 0.5% glucose and 5 mg/mL chloramphenicol at 30°C, or on M17 agar plates with the same supplements.

Antibody production. Rabbit polyclonal antibodies were raised at Eurogentec (Seraing, Liège, Belgium) for detection of PPAD, Omp41, Kgp, and RgpA + RgpB. To this end, the PPAD gene from reference strain W83 was cloned and expressed without its signal sequence in *Lactococcus lactis* PA1001[31,32], using plasmid pNG4210[33,34] and primers P1LF and P1LR (S2 Table). To raise Omp41-specific antibodies, the *omp41* gene of strain W83 was amplified with primers P1F and P1R (S2 Table). To raise Kgp-specific antibodies, the region of the *kgp* gene from *P. gingivalis* ATCC 33277 encoding the catalytic domain was amplified with primers P2F and P2R (S2 Table). To raise antibodies recognizing both RgpA and RgpB (hereafter named RgpA/B), the region of the *rgpA* gene from *P. gingivalis* ATCC 33277 encoding the catalytic domain of RgpA that is nearly identical to the catalytic domain of RgpB[35,36], was amplified with primers P3F and P3R (S2 Table). The resulting *omp41, kgp, rgpA/ B* fragments were all cloned and expressed in *E. coli* BL21 using pET26B^{+}. Expression of PPAD in *L. lactis* was induced overnight with nisin as described[33], while expression of Omp41 or the Kgp and RgpA catalytic domains in *E. coli* was induced with Isopropyl-b-D-thio-galactopyranoside for 2 h. After induction, cells were collected by centrifugation and disrupted by sonication (*L. lactis*) or bead-beating (*E. coli*) in binding buffer (S3

Table). Cell lysates were added to 500 mL of HisLinkTM Protein Purification Resin (Promega, USA) and incubated at room temperature for 30–60 min. To remove non-specifically bound proteins, the resin was washed thrice with 10 mL wash buffer (S3 Table) and transferred to a column. Elution was performed by collecting four fractions: two with 1.6 mL of elution buffer 1 and two with 1.6 mL of elution buffer 2 (S3 Table). The eluted proteins in immunization buffer (S3 Table) were used to immunize rabbits. Of note, RgpA/B antibodies binding the catalytic domain of RgpA will also bind RgpB, but the two proteins can be distinguished by virtue of their different molecular weights.

Analysis of secreted proteins. Proteins secreted into the culture medium (2 mL) were collected through 10% TCA precipitation as described[30]. Subsequently, they were separated by LDS-PAGE (NuPAGE gels, Life Technologies) [30]. Western blotting analysis was performed using AmershamTM Protran® 0.45 mm nitrocellulose membranes (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and antibodies specific for PPAD (GP2448), Omp41 (GP2451), or gingipains. After overnight incubation with 5% skim milk in phosphate-buffered saline (PBS), membranes were washed with PBS-Tween20 (PBS-T) and incubated with primary anti-PPAD, Omp41, Kgp or RgpA/B antibodies in a 1:10000 dilution. Following one-hour incubation, membranes were washed four times for 5 min with PBS-T before being incubated for 45 min with a goat anti-rabbit secondary antibody IRDye® 800CW conjugate (LI-COR Biosciences, Lincoln, NE, USA) at a 1:10000 dilution. Afterwards, membranes were washed four times in PBS-T and twice in PBS, before scanning with an Odyssey Infrared Imaging System (LI-COR Biosciences).

Collection of OMVs. To collect OMVs, 2 mL bacterial culture aliquots were centrifuged at 16100 x *g*, 4°C, for 20 min to separate cells from OMVs in the growth medium. 500 mL aliquots of the resulting supernatant were subjected to ultracentrifugation at 213000 x *g*, 4°C, for 2 h in an Optima MAX-XP ultracentrifuge (Beckman Coulter, Brea, CA, USA) using an MLA-80 fixed angle rotor. The resulting pellet containing OMVs was resuspended in 500 mL of PBS with 5 mM $MgCl_2$. When needed, the vesicle fraction was concentrated by precipitation with 10% TCA.

Protease inhibition. Proteolytic activity was inhibited by growing bacteria in presence of the cOmplete Mini or cOmplete EDTA-free protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany), according to manufacturer's specifications.

Transmission electron microscopy (TEM) Bacteria. 2 mL bacterial culture aliquots were centrifuged at 16100 x *g*, 4°C, for 20 min. The resulting pellets were resuspended in pre-fixative (S3 Table) for 20 min at room temperature. Subsequently, bacteria were pelleted again and resuspended in fixative (S3 Table) for 2 h. After washing with 0.1 M sodium cacodylate, bacteria were pelleted in 2% low-melting-point agarose. After 2 h, 4°C treatment with post-fixative (S3 Table), bacteria were dehydrated using ethanol and embedded in EPON resin (Serva, Heidelberg, Germany). 60 nm sections were cut with an ultramicrotome UC7 (Leica, Vienna, Austria) and contrasted using 2% uranyl acetate, followed

by Reynolds lead citrate. Images were recorded with a FEI Cm100 transmission electron microscope operated at 80 KV using a Morada digital camera.

OMVs. OMVs were collected from 8 mL bacterial cultures as indicated above. Upon ultracentrifugation, pellets were resuspended in 20 µL PBS. When needed, these samples were further diluted 1:50. 10 µL of vesicle suspension were placed on Formvar coated TEM grids. After 10 min, liquid was drained using filter paper and grids were placed upside down onto drops of 2% ammonium molybdate for 2 min, and transferred to a drop of water for 30 sec. Subsequently, liquid was drained with filter paper and grids were air-dried before TEM examination as specified above.

Sequence analyses. Total DNA from the nine *P. gingivalis* sorting type II strains was sequenced as described[28]. PPAD gene sequences were retrieved from the nine assembled genomes, seven previously sequenced *P. gingivalis* genomes[28], and 15 *P. gingivalis* genomes in GenBank (S4 Table). PPAD genes and their deduced amino acid sequences were aligned using the MAFFT v7 web server[37]. The sequence reads obtained from whole genome sequencing were submitted to the European Nucleotide Archive under project PRJEB20287 with accession numbers: ERS1718891, ERS1718892, ERS1718893, ERS1718894, ERS1718895, ERS1718896, ERS1718897, ERS1718898, and ERS1718899.

Modeling of PPAD three-dimensional (3D) structures. 3D structures of PPAD were initially modeled by homology through the online server SWISS-MODEL[38]. To consider possible steric changes in the proteins due to point mutations, geometrical optimizations of the structures were performed using Hyperchem V.8[39]. This minimization of energy was performed *ab initio* with the Polak-Ribiere optimization method and 0.1 kcal/(Å•mol) as termination parameter. Quality assessment of final structures was achieved using QMEAN[40] and PRO- CHECK[41]. Visualization and localization of substitutions were performed in Pymol[42]. To acquire the electrostatic potential surface, Poisson-Boltzmann electrostatics calculations were applied to optimized PDB structures using an AMBER force field and the PDB2PQR server[43].

Ethics statement

The bacterial samples used in the present analyses were obtained in a previous study upon written informed consent[28]. This previous study received Institutional Review Board approval from the Medical Ethics Committee of the University Medical Center Groningen (METc UMCG 2011/010). It was performed in accordance with the guidelines of the Declaration of Helsinki and the institutional regulations, and all samples were anonymized.

Disclosure statement

The authors declare that they have no financial and non-financial competing interests in relation to the documented research.

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Supplementary Material

Table S1. Panel of the bacterial isolates analyzed, including original disease, severity of periodontitis (PD) and general health condition of the host.

Table S2. List of primers used in the cloning procedures.

Table S3. List of all the buffers used in the cloning procedures and preparation of samples for the transmission electron microscopy (TEM) analysis.

Table S4. List of all the published sequences and genomes of the *P. gingivalis* strains used in the mutation analysis and relative NCBI accession codes.

Table S5. Amino acid substitutions in the PPAD sequence of *P. gingivalis* sorting type II isolates, reference strains of known and unknown sorting status, previously sequenced clinical isolates (sorting type I), and published genomes with unknown PPAD sorting status.

Figure S1. Western blot analysis of the presence of PPAD in growth medium samples from *P. gingivalis* reference strains, PPAD mutants, and sorting type I and II isolates.

Figure S2. Western blot analysis of PPAD presence in the cell fractions of sorting type I and II isolates.

Figure S3. Western blot analysis of the presence of Omp41 in growth medium samples of *P. gingivalis* reference strains, and sorting type I and II isolates.

Figure S4. Western blot analysis of the presence of (A) RgpA/B and (B) Kgp in growth medium samples of *P. gingivalis* reference strains, and sorting type I and II isolates.

Figure S5. Western blot analysis of growth medium (supernatant) samples of *P. gingivalis* sorting type II isolates with or without exposure to different protease inhibitors.

References

1. McGraw, W. T., Potempa, J., Farley, D. & Travis, J. Purification, characterization, and sequence analysis of a potential virulence factor from *Porphyromonas gingivalis*, peptidylarginine deiminase. *Infect. Immun.* 67, 3248–56 (1999).
2. Gui, M. J., Dashper, S. G., Slakeski, N., Chen, Y.-Y. & Reynolds, E. C. Spheres of influence: *Porphyromonas gingivalis* outer membrane vesicles. *Mol. Oral. Microbiol.* 31, 365–378 (2016).
3. Yang, H.-W., Huang, Y.-F. & Chou, M.-Y. Occurrence of *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontally diseased and healthy subjects. *J. Periodontol.* 75, 1077–1083 (2004).
4. Quirke, A.-M. *et al.* Heightened immune response to autocitrullinated *Porphyromonas gingivalis* peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis. *Ann. Rheum. Dis.* 73, 263–269 (2014).
5. de Pablo, P., Dietrich, T. & McAlindon, T. E. Association of periodontal disease and tooth loss with rheumatoid arthritis in the US population. *J. Rheumatol.* 35, 70–76 (2008).
6. Detert, J., Pischon, N., Burmester, G. R. & Buttgereit, F. The association between rheumatoid arthritis and periodontal disease. *Arthritis Res. Ther.* 12, 218 (2010).
7. de Smit, M. *et al.* Periodontitis in established rheumatoid arthritis patients: a cross-sectional clinical, microbiological and serological study. *Arthritis Res. Ther.* 14, R222 (2012).
8. de Smit, M. J., Brouwer, E., Vissink, A. & van Winkelhoff, A. J. Rheumatoid arthritis and periodontitis; a possible link via citrullination. *Anaerobe* 17, 196–200 (2011).
9. de Smit, M. J. *et al.* [Effect of periodontal treatment on rheumatoid arthritis and vice versa]. *Ned. Tijdschr. Tandheelkd.* 119, 191–197 (2012).
10. Laroche, D., Pozzo, T., Ornetti, P., Tavernier, C. & Maillefert, J. F. Effects of loss of metatarsophalangeal joint mobility on gait in rheumatoid arthritis patients. *Rheumatology (Oxford)* 45, 435–440 (2006).
11. Lee, D. M. & Weinblatt, M. E. Rheumatoid arthritis. *Lancet* 358, 903–911 (2001).
12. Pischon, N. *et al.* Association among rheumatoid arthritis, oral hygiene, and periodontitis. *J. Periodontol.* 79, 979–986 (2008).
13. Routsias, J. G., Goules, J. D., Goules, A., Charalampakis, G. & Pikazis, D. Autopathogenic correlation of periodontitis and rheumatoid arthritis. *Rheumatology (Oxford)* 50, 1189–93 (2011).
14. Mangat, P., Wegner, N., Venables, P. J. & Potempa, J. Bacterial and human peptidylarginine deiminases: targets for inhibiting the autoimmune response in rheumatoid arthritis? *Arthritis Res. Ther.* 12**,** 209 (2010).
15. Avouac, J., Gossec, L. & Dougados, M. Diagnostic and predictive value of anti-cyclic citrullinated protein antibodies in rheumatoid arthritis: a systematic literature review. *Ann. Rheum. Dis.* 65, 845–851 (2006).
16. Nishimura, K. *et al.* Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. *Ann. Intern. Med.* 146, 797–808 (2007).
17. Sato, K. *et al.* Identification of *Porphyromonas gingivalis* proteins secreted by the Por secretion system. *FEMS Microbiol. Lett.* 338, 68–76 (2013).
18. Glew, M. D. *et al.* PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. *J. Biol. Chem*. 287, 24605–24617 (2012).
19. McBride, M. J. & Zhu, Y. Gliding motility and Por secretion system genes are widespread among members of the phylum bacteroidetes. *J. Bacteriol*. 195, 270–278 (2013).
20. Shoji, M. *et al.* Por secretion system-dependent secretion and glycosylation of *Porphyromonas gingivalis* hemin-binding protein 35. *PLoS ONE* 6, e21372 (2011).
21. Lasica, A. M., Ksiazek, M., Madej, M. & Potempa, J. The Type IX Secretion System (T9SS): Highlights and Recent Insights into Its Structure and Function. *Front. Cell Infect. Microbiol.* 7, 215 (2017).
22. Veith, P. D., Glew, M. D., Gorasia, D. G. & Reynolds, E. C. Type IX secretion: the generation of bacterial cell surface coatings involved in virulence,

gliding motility and the degradation of complex biopolymers. *Mol. Microbiol.* 106, 35–53 (2017).

23. Xie, H. Biogenesis and function of *Porphyromonas gingivalis* outer membrane vesicles. *Future Microbiol.* 10, 1517–1527 (2015).

24. Veith, P. D. *et al. Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J. Proteome Res.* 13, 2420–2432 (2014).

25. Konig, M. F., Paracha, A. S., Moni, M., Bingham, C. O. & Andrade, F. Defining the role of *Porphyromonas gingivalis* peptidylarginine deiminase (PPAD) in rheumatoid arthritis through the study of PPAD biology. *Ann. Rheum. Dis.* 74, 2054–61 (2014).

26. Shoji, M. *et al.* Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains. *Microbiology (Reading, Engl.)* 148, 1183–1191 (2002).

27. Ho, M.-H., Chen, C.-H., Goodwin, J. S., Wang, B.-Y. & Xie, H. Functional Advantages of *Porphyromonas gingivalis* Vesicles. *PLoS ONE* 10, e0123448 (2015).

28. Gabarrini, G. *et al.* The peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis*. *Sci. Rep.* 5, 13936 (2015).

29. Montgomery, A. B. *et al.* Crystal structure of *Porphyromonas gingivalis* peptidylarginine deiminase: implications for autoimmunity in rheumatoid arthritis. *Ann. Rheum. Dis.* 75**,** 1255–1261 (2016).

30. Stobernack, T. *et al.* Extracellular Proteome and Citrullinome of the Oral Pathogen *Porphyromonas gingivalis*. *J. Proteome Res.* 15, 4532–4543 (2016).

31. Wegner, N. *et al.* Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. *Immunol. Rev.* 233, 34–54 (2010).

32. Neef, J., Koedijk, D. G. A. M., Bosma, T., van Dijl, J. M. & Buist, G. Efficient production of secreted staphylococcal antigens in a non-lysing and proteolytically reduced Lactococcus lactis strain. *Appl. Microbiol. Biotechnol.* 98, 10131–10141 (2014).

33. Hoekstra, H. *et al.* A human monoclonal antibody that specifically binds and inhibits the staphylococcal complement inhibitor protein SCIN. *Virulence* 9, 70–82 (2018).

34. Neef, J. *et al.* Versatile vector suite for the extracytoplasmic production and purification of heterologous His-tagged proteins in Lactococcus lactis. *Appl. Microbiol. Biotechnol.* 99, 9037–9048 (2015).

35. Mikolajczyk-Pawlinska, J. *et al.* Genetic variation of *Porphyromonas gingivalis* genes encoding gingipains, cysteine proteinases with arginine or lysine specificity. *Biol. Chem.* 379, 205–211 (1998).

36. Olczak, T., Simpson, W., Liu, X. & Genco, C. A. Iron and heme utilization in *Porphyromonas gingivalis*. *FEMS Microbiol. Rev.* 29, 119–144 (2005).

37. Katoh, K., Misawa, K., Kuma, K.-I. & Miyata, T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30, 3059–3066 (2002).

38. Biasini, M. *et al.* SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* 42, W252–8 (2014).

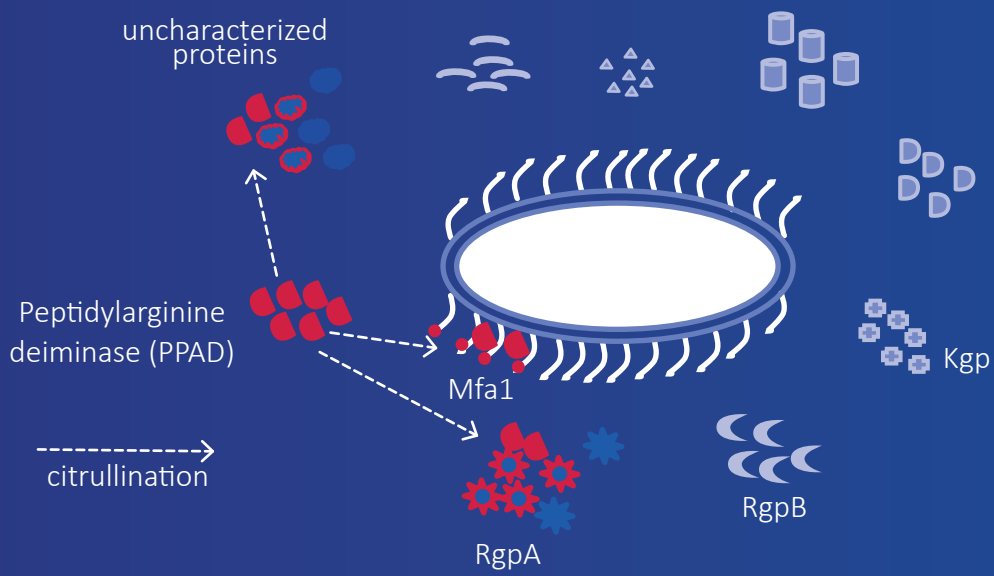
39. Froimowitz, M. HyperChem: a software package for computational chemistry and molecular modeling. *BioTechniques* 14, 1010–1013 (1993).

40. Benkert, P., Künzli, M. & Schwede, T. QMEAN server for protein model quality estimation. *Nucleic Acids Res.* 37, W510–4 (2009).

41. Laskowski, R. A., Rullmannn, J. A., MacArthur, M. W., Kaptein, R. & Thornton, J. M. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* 8, 477–486 (1996).

42. Schrodinger,, LLC. The PyMOL Molecular Graphics System, Version 1.8. (2015).

43. Dolinsky, T. J., Nielsen, J. E., McCammon, J. A. & Baker, N. A. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res.* 32, W665–7 (2004).


uncharacterized proteins
Peptidylarginine deiminase (PPAD)
citrullination
Mfa1
RgpA
RgpB
Kgp

CHAPTER 4

Extracellular proteome and citrullinome of the oral pathogen *Porphyromonas gingivalis*

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Abstract

Porphyromonas gingivalis is an oral pathogen associated with the inflammatory disease periodontitis. Periodontitis and *P. gingivalis* have been associated with rheumatoid arthritis. One of the hallmarks of rheumatoid arthritis is the loss of tolerance against citrullinated proteins. Citrullination is a post-translational modification of arginine residues, leading to a change in structure and function of the respective protein. This modification, which is catalysed by peptidylarginine deiminases (PAD), plays a role in several physiological processes in the human body. Interestingly, *P. gingivalis* secretes a citrullinating enzyme, known as *P. gingivalis* PAD (PPAD), which targets bacterial and human proteins. Since the extent of *P. gingivalis* protein citrullination by PPAD was not yet known, the present study was aimed at identifying the extracellular proteome and citrullinome of *P. gingivalis*. To this end, extracellular proteins of two reference strains, two PPAD-deficient mutants and three clinical isolates of *P. gingivalis* were analysed by mass spectrometry. The results uncovered substantial heterogeneity in the extracellular proteome and citrullinome of *P. gingivalis*, especially in relation to the extracellular detection of typical cytoplasmic proteins. In contrast, the major virulence factors of *P. gingivalis* were identified in all investigated isolates although their citrullination was shown to vary. This may be related to post-translational processing of the PPAD enzyme. Altogether, our findings focus attention on the possible roles of six to 25 potentially citrullinated proteins, especially the gingipain RgpA, in periodontitis and rheumatoid arthritis.

Key words: *Porphyromonas gingivalis*, protein sorting, exoproteome, citrullination, peptidylarginine deiminase

Abbreviations used are the following: ACPAs, anti-citrullinated protein antibodies; BA2, Blood Agar Base No. 2; BHI, brain heart infusion; BSL-2, biosafety level 2; CID, collision induced dissociation; ESI, electrospray ionization; GO, gene ontology; IAA, iodoacetamide; LC, liquid chromatography; LDS, lithium dodecyl sulphate; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; MS, mass spectrometry; OD, optical density at 600 nm; PAD, peptidylarginine deiminase PPAD, *Porphyromonas gingivalis* peptidylarginine deiminase; RA, rheumatoid arthritis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid

Introduction

Periodontitis is an inflammatory disease affecting the soft and hard tissues surrounding the teeth. It is primarily caused by bacterial deposits organized in a subgingival biofilm. Severe periodontitis has a prevalence of 10 – 15% in the general adult population and is the major cause for tooth loss over the age of 35[1,2]. Subgingival carriage of *Porphyromonas gingivalis* has been implicated in the development of periodontitis in susceptible hosts[3-6]. Interestingly, *P. gingivalis* and periodontitis have both been associated with systemic diseases like rheumatoid arthritis (RA)[2,7-9]. RA is a chronic inflammation of synovial joints with a prevalence of 0.5 - 1.0% in the general population. A specific feature of RA development is the loss of tolerance to citrullinated proteins. This correlates with the formation of anti-citrullinated protein antibodies (ACPAs) that seem to trigger a preclinical state of the disease[2,8,10,11] and are associated with a poorer disease outcome or increased joint damage and low remission rates[12].

Citrullination is a post-translational protein modification where L-arginine is enzymatically converted to L-citrulline, which causes changes in the charge and structure of the respective target proteins[13]. In the human body, citrullination occurs during cell apoptosis and it is an important factor in skin keratinization, insulation of neurons, general development of the central nervous system, and various gene regulatory mechanisms[13-16]. Citrullination is catalyzed by peptidylarginine deiminases (PADs), of which five isotypes have been identified in humans. An analogous enzyme is found in *P. gingivalis*. To date, *P. gingivalis* is the only bacterium known to produce a PAD enzyme[17-19]. Studies have shown that the *P. gingivalis* PAD (PPAD) citrullinates not only bacterial but also human proteins (e.g. α-enolase and vimentin). This mechanism of human protein citrullination by PPAD may be a connection between periodontitis and RA[11,13].

P. gingivalis is a Gram-negative bacterium. Accordingly, it has a cell envelope consisting of two distinct membranes, the inner membrane and the outer membrane, which enclose the periplasm. Thus, at least five sub-proteomes can be distinguished, specifically the cytoplasmic, inner membrane, periplasmic, outer membrane and exoproteome. The outer membrane acts as a permeation barrier that serves in the cellular retention of periplasmic proteins. Many virulence factors of Gram-negative bacteria are located in/on the outer membrane or are secreted. Important proteinaceous virulence factors of *P. gingivalis* are fimbriae and gingipains. The fimbriae are appendages that enable this pathogen to attach to other bacteria or to host cells, which is important in biofilm formation. Gingipains are cysteine proteinases and account for 85% of the total proteolytic activity of *P. gingivalis*[4,20].

Notably, the secreted PPAD can be regarded as another virulence factor that may impact on the host's immune system by increasing the level of citrullinated proteins. This could lead to a loss of tolerance against citrullinated proteins and the subsequent development of RA, which raises the question to what extent PPAD citrullinates the proteins that *P. gingivalis* secretes into its extracellular milieu. Conceivably, such citrullinated bacterial proteins could contribute to the overall citrullination burden in the human host. Several previous studies investigated the overall proteome of *P. gingivalis* exposed to different conditions[21-27]. However, to date very little is known about the exoproteome composition of especially clinical isolates of *P. gingivalis* and the extent to which the respective proteins

are citrullinated. Therefore, the aim of this study was to define the exoproteome of *P. gingivalis* and to investigate the possible citrullination of the identified proteins by mass spectrometry (MS). To this end, different *P. gingivalis* reference strains including two PPAD mutants as well as clinical isolates were investigated. This allowed a definition of the core and variable exoproteomes. Importantly, a number of citrullinated exoproteins of *P. gingivalis* was identified, which are collectively referred to as the *P. gingivalis* 'citrullinome'.

Material and Methods

***P. gingivalis isolates* used in this study.** The *P. gingivalis* reference strains ATCC 33277 and W83, as well as two respective PPAD-deficient mutants[18] were used. Furthermore, three previously described clinical isolates were analysed[17], which had been obtained from a patient with severe periodontitis without RA (20658), a patient with moderate periodontitis and RA (MDS16), and a patient with severe periodontitis and RA (MDS45).

Growth conditions. Bacteria were grown anaerobically either on Blood Agar Base No. 2 (BA2) plates, or in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) that contained 5% (w/v) L-cysteine, 5 mg/L hemin and 1 mg/L menadione. Prior to inoculation, the BHI broth was pre-reduced for 3 days in an anaerobic chamber.

To start liquid cultures, 5-day old colonies from BA2 plates were inoculated anaerobically into serum bottles with a rubber septum in the cap that contained 30 mL of BHI broth. The colonies were then dispersed in the BHI broth using a syringe with a 21G needle. Lastly, the bottles were tightly closed and anaerobically incubated at 37°C. For proteome analyses, culture samples were collected in the stationary phase after 24 to 32 h of growth using a syringe with a 21G needle.

Species verification. *P. gingivalis* colonies grown on BA2 plates were verified by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS, using a MALDI Biotyper® (Bruker Corporation, Billerica, USA). Briefly, one single colony was spotted twice on the MALDI target of a mass spectrometer. Next, 1 µL extraction buffer (containing formaldehyde) was added to each spot. After ~25 min incubation, 1 µL of matrix material (α-Cyano-4-hydroxycinnamic acid, Sigma-Aldrich, St. Louis, USA) was added to each spot and MS spectra were recorded. A minimal score value of 2.0 was used as a criterion for *P. gingivalis* identification[28].

Preparation of exoproteome samples. To analyze the exoproteome of *P. gingivalis*, cells were grown in triplicates in liquid culture until early stationary phase (approx. 24-32h of growth). 2 mL of culture was centrifuged for 10 min at 8.000 x g and 4°C. 1.2 mL of the supernatant was added to 0.3 mL of 50% trichloroacetic acid (TCA, Sigma-Aldrich, St. Louis, USA), mixed thoroughly and stored on ice at 4°C overnight for precipitation of proteins. In order to collect the precipitated proteins, the sample was

centrifuged for 20 min at 13.200 x g and 4°C. After one washing step with 500 µL ice-cold pure acetone, the pellet was collected again by centrifugation for 10 min at 14.000 rpm and 4°C. The acetone was removed and the protein pellet was dried at room temperature or 60°C and stored at -20°C until further use.

LDS-PAGE. Lithium dodecyl sulphate (LDS) PAGE was performed using 10% NuPAGE gels (Invitrogen, Carlsbad, USA). Cells were resuspended in LDS buffer (Life Technologies) and disrupted by bead-beating with 0.1 µm glass beads (Biospec Products, Bartlesville, USA) using a Precellys24 (Bertin Technologies, Montigny-le-Bretonneux, France), and exoproteins were precipitated from the growth medium with 10% TCA (4°C, overnight). Protein samples were incubated for 10 min at 95ºC, separated by LDS-PAGE, and stained with SimplyBlue™ SafeStain (Life Technologies, Carlsbad, USA).

Sample preparation for mass spectrometry. Dried exoproteome pellets were first digested following an in-solution trypsin digestion procedure. The whole-protein pellets were resuspended in 100 µL of 50 mM ammonium bicarbonate buffer (Fluka, Buchs, Switzerland). The samples were reduced by addition of 2 µL of 500 mM DTT and incubation for 45 min at 60°C. Subsequently, the samples were alkylated by addition of 2 µL of 500 mM iodoacetamide (IAA, Sigma-Aldrich, St. Louis, USA), and incubated in the dark for 15 min at room temperature. Trypsin (80 ng; Promega, Madison, USA) was added and samples were incubated overnight at 37°C under continuous shaking at 250 rpm. The trypsin digestion was stopped by addition of 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, St. Louis, USA) and incubation at 37°C for 45 min.

To purify in-solution digested peptides, a ZipTip® (Millipore, Billerica, USA) filtration procedure was implemented as described by Dreisbach *et al.*[29]. Briefly, after wetting and equilibrating a ZipTip® pipette tip, the peptides were bound to C-18 material. A subsequent washing step with 0.1% acetic acid was followed by a final elution with 60% acetonitrile and 0.1% acetic acid. The samples were dried at room temperature in a Concentrator Plus Speed Vac (Eppendorf, Hamburg, Germany) using the V-AQ program, and the resulting peptide pellets were stored at 4°C until further use.

Mass spectrometry analysis. Purified peptides were analyzed by reversed phase liquid chromatography (LC) electrospray ionization (ESI) MS/MS using an LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA) as described by Bonn *et al.*[30]. In brief, in-house self-packed nano-LC columns (20 cm) were used to perform LC with an EASY-nLC II system (Thermo Fisher Scientific, Waltham, USA). The peptides were loaded with buffer A (0.1 % acetic acid (v/v)) and subsequently eluted by a binary gradient of buffer A and B (0.1 % acetic acid (v/v), 99.9 % acetonitrile) over a period of 80 min. After injection into the MS, a full scan was recorded in the Orbitrap with a resolution of 30,000. The five most abundant precursor ions were consecutively isolated in the LTQ XL and fragmented via collision-induced dissociation (CID). Unassigned charge states as well as singly charged ions were rejected and the lock mass option was enabled.

Database searching was done with Sorcerer-SEQUEST 4 (Sage-N Research, Milpitas, USA). After extraction from the raw files, *.dta files were searched with Sequest against a target-decoy database with a set of common laboratory contaminants. A non-redundant database for the respective peptide/protein search was created from the published genome sequences of the W83, ATCC 33277 and TDC60 strains which were downloaded from Uniprot (http://www.uniprot.org) on 21/10/2014 (Supplementary FASTA file). The created database contained a total number of 12254 proteins. Protein sequences that differed in only 1 amino acid were included in this database. Of note, some poorly conserved proteins of the clinical isolates will be missing from the database, because their genome sequences have not been determined. Database search was based on a strict trypsin digestion with two missed cleavages permitted. No fixed modifications were considered. Oxidation of methionine, carbamidomethylation of cysteine and citrullination of arginine were considered as variable modifications. The mass tolerance for precursor ions was set to 10 ppm and the mass tolerance for fragment ions to 0.5 Da. Validation of MS/MS-based peptide and protein identification was performed with Scaffold v.4.4.1.1 (Proteome Software, Portland, USA). Peptide identifications were accepted if they exceeded the following specific database search engine thresholds. SEQUEST identifications required at least deltaCn scores of greater than 0.1 and XCorr scores of greater than 2.2, 3.3 and 3.75 for doubly, triply and all higher charged peptides, respectively. Protein identifications were accepted if at least 2 identified peptides were detected with the above-mentioned filter criteria in 2 out of 3 biological replicates. With these filter parameters, no false-positive hits were obtained, as was verified by a search against a concatenated target-pseudoreversed decoy database. However, it should be noted that these filter parameters can potentially lead to false-negative hits, especially in the case of low-abundant proteins. Thus, if a protein is not identified, this does not necessarily mean that it is not present at all.

Protein data were exported from Scaffold and further curated in Microsoft Excel 2010 before further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository[31] with the dataset identifier PXD003444.

Quantitative values of protein abundances were obtained by summing up all spectra associated with a specific protein within a sample which includes also those spectra that are shared with other proteins. To allow comparisons, spectral counts were normalized by applying a scaling factor for each sample to each protein adjusting the values to normalized spectral counts. Of note, some proteins are easier to detect than others, which may affect the comparison of abundance levels of different proteins.

Citrullination of proteins was detected by a mass shift of 1 Dalton in arginine-containing peptides. To exclude false-positive identifications, peptides containing asparagine and/or glutamine were excluded from this analysis, because it is not possible to distinguish between citrullination of arginine, and deamidation of asparagine or glutamine. The spectra and fragmentation Tables of six identified citrullinated proteins are shown in Supplementary Figure S6.

Statistical analyses. Statistical analyses of the relative exoprotein abundances were performed as follows. Replicate values of the normalized total spectral counts were imported into GraphPad Prism 6 (GraphPad Software, La Jolla, USA). A Two-Way ANOVA Turkey's multiple comparison test was

performed, where the mean exoprotein abundance values of every bacterial isolate were compared with each other within every single exoprotein row in the respective heatmap. This led to the detection of simple effects within each exoprotein row. The total number of significant differences within the 20 most abundant and within the 50 least abundant exoproteins was used as a measure of heterogeneity in the respective fractions of the bacterial exoproteomes.

Bioinformatic analyses. Protein localization predictions were performed using the following algorithms: LipoP (version 1.0)[32], Lipo (version 1.0)[33], TMHMM (version 2.0)[34,35], Phobius (version 1.0)[36], SignalP (version 4.1)[37], Predisi (version 1.0)[38], SecretomeP (version 2.0)[39], PsortB (version 3.0)[40] and ClubSub (version 2.18.3)[41]. Furthermore, manual curation based on *Bacteroidetes/Porphyromonas*-specific domain identification[54] was done for proteins with unclear localization predictions.

For visualization of protein functions, the gene ontology (GO) terms of the present protein dataset were imported into the REVIGO software[42].

Biological and chemical safety. *P. gingivalis* is a biosafety level 2 (BSL-2) microbiological agent and was accordingly handled following appropriate safety procedures. All experiments involving live *P. gingivalis* bacteria and chemical manipulations of *P. gingivalis* protein extracts were performed under appropriate containment conditions, and protective gloves were worn. All chemicals and reagents used in this study were handled according to the local guidelines for safe usage and protection of the environment.

Results

Exoproteomes of *P. gingivalis* reference strains and clinical isolates

A total of seven *P. gingivalis* reference strains and clinical isolates were examined in the present study. Growth experiments in BHI broth revealed some differences in growth rates and maximal optical density (OD) at 600 nm, especially between the reference strains and the clinical isolates. As shown in Supplementary Figure S1, the reference strains displayed higher growth rates while reaching lower maximum ODs. Since only few proteins were found to be secreted by exponentially growing cells compared to stationary phase cells (Supplementary Figure S2), exoproteome analyses were only performed on early stationary phase samples. Further, as shown by LDS-PAGE (Figure S2), the different isolates revealed different protein banding patterns, suggesting isolate-specific variations in their exoproteome composition. Based on these observations, the exoproteome fractions of the different *P. gingivalis* isolates were further analyzed by the gel-free proteomics approach LC-ESI-MS/MS. A total number of 257 proteins was identified in the combined exoproteome of the seven different isolates (Supplementary Table S1). The total numbers of proteins identified per isolate ranged from 124 to 202 proteins (Supplementary Figure S3A). Remarkably, 202 extracellular proteins were identified for the MDS45 isolate, while for all other isolates between 124-147 extracellular proteins were identified.

Figure 1 illustrates the overlaps and differences in the exoproteomes of the investigated isolates. While the two reference strains W83 and ATCC 33277 had 83 proteins in common, they also expressed a high number of proteins that were not shared by these two strains (60 and 41 respectively; Supplementary Figure S3B). Remarkably, the exoproteomes of the clinical isolates seemed more conserved with 115 common proteins. For the isolates 20658 and MDS16 three and 13 unique proteins were identified, respectively, while 60 unique proteins were identified for MDS45 (Supplementary Figure S3C).

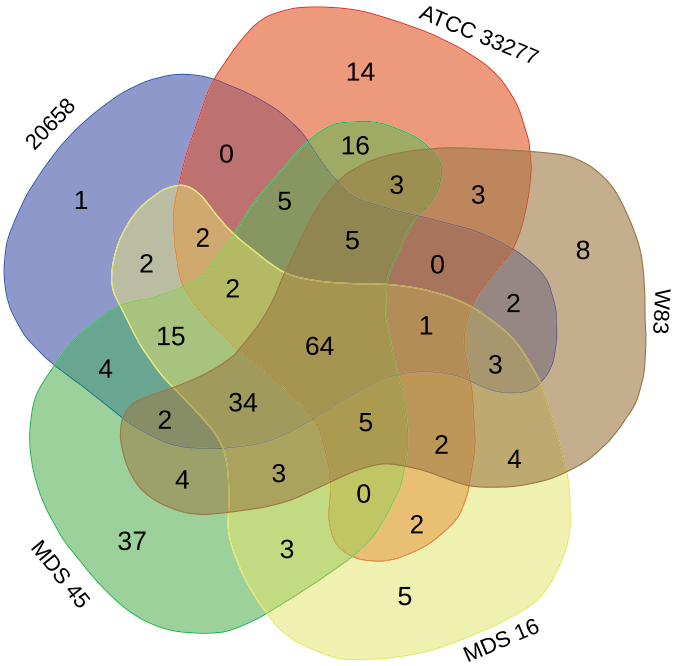


Figure 1: Differential detection of proteins in the exoproteomes of the *P. gingivalis* isolates. Venn diagram giving an overview of the numbers of consistently or uniquely identified proteins in the two reference strains W83 and ATCC 33277 and the three clinical isolates 20658, MDS16 and MDS45. The diagram was created using the Venn diagram web tool of the VIB and the University of Gent in Belgium (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Protein localization predictions reveal differences between the core and variable exoproteomes

With the implementation of different bioinformatic tools and a manual curation based on domain identification, the subcellular localization of the identified exoproteins was predicted. The results are presented in Figure 2 and Supplementary Figure S4, showing a rather homogeneous pattern for the different isolates with a dominant fraction of predicted outer membrane proteins and a marginal fraction of predicted inner membrane proteins amongst the identified exoproteins. The largest differences were observed in the relative amounts of predicted periplasmic and cytoplasmic proteins. Specifically, the predicted cytoplasmic proteins detected in the growth media of the W83, MDS16 and 20658 isolates represented ~15-20% of the whole exoproteome, while these proteins represented ~20-30% in the media of the remaining isolates. Conversely, the predicted periplasmic proteins detected for the W83, MDS16 and 20658 isolates represented ~15-20% of the whole exoproteome, while the proportion of predicted periplasmic proteins was ~10-15% for the other isolates.

In a next step, the core and variable exoproteomes of *P. gingivalis* were defined based on the presently collected data. PPAD-deficient mutants were excluded from this analysis, because they were genetically engineered, whereas the PPAD gene is present in all clinical *P. gingivalis* isolates[17]. The identified core exoproteome includes 64 proteins that were found for the three clinical isolates and the two reference strains. All remaining 193 proteins belong to the variable exoproteome, meaning that they were not detectable in all of these five isolates. A list of the proteins assigned to the core and variable exoproteomes is presented in the Supplementary Table S1. The predicted localization of the respective proteins suggests that the core exoproteome includes a very high proportion of extracellular and outer membrane proteins (23.5% and 48.5% respectively; Figure 2A). Almost no inner membrane proteins (1.5%) and relatively low numbers of predicted cytoplasmic (12.5%) and periplasmic (14%) proteins were found in the core exoproteome. In contrast, the variable exoproteome was found to include a significantly higher proportion of predicted cytoplasmic proteins (33%) and inner membrane proteins (4%), while the proportions of predicted extracellular and outer membrane proteins was lower (14% and 33% respectively) compared to the core exoproteome. Considering the complete set of identified extracellular proteins, the largest proportion of proteins is predicted to be outer membrane proteins (37%). About 28% of the extracellular proteins are predicted to be cytoplasmic, 16% extracellular, 15.5% periplasmic and 3.5% inner membrane proteins (Figure 2B).

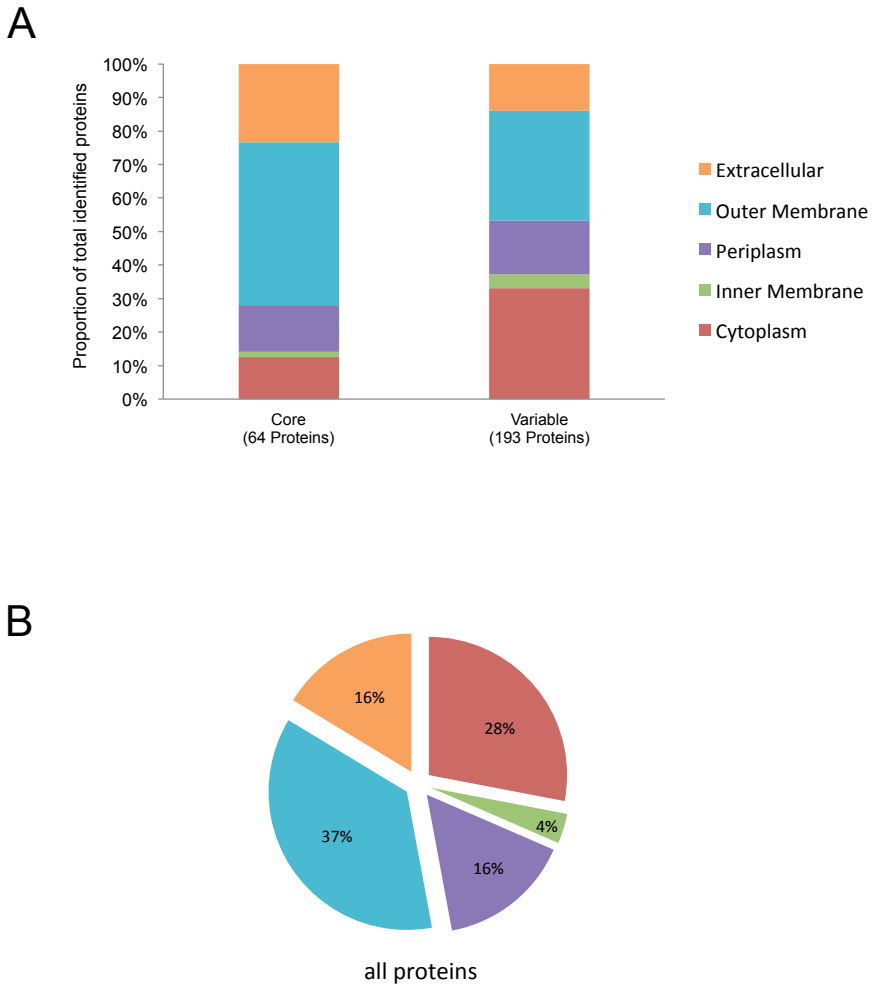
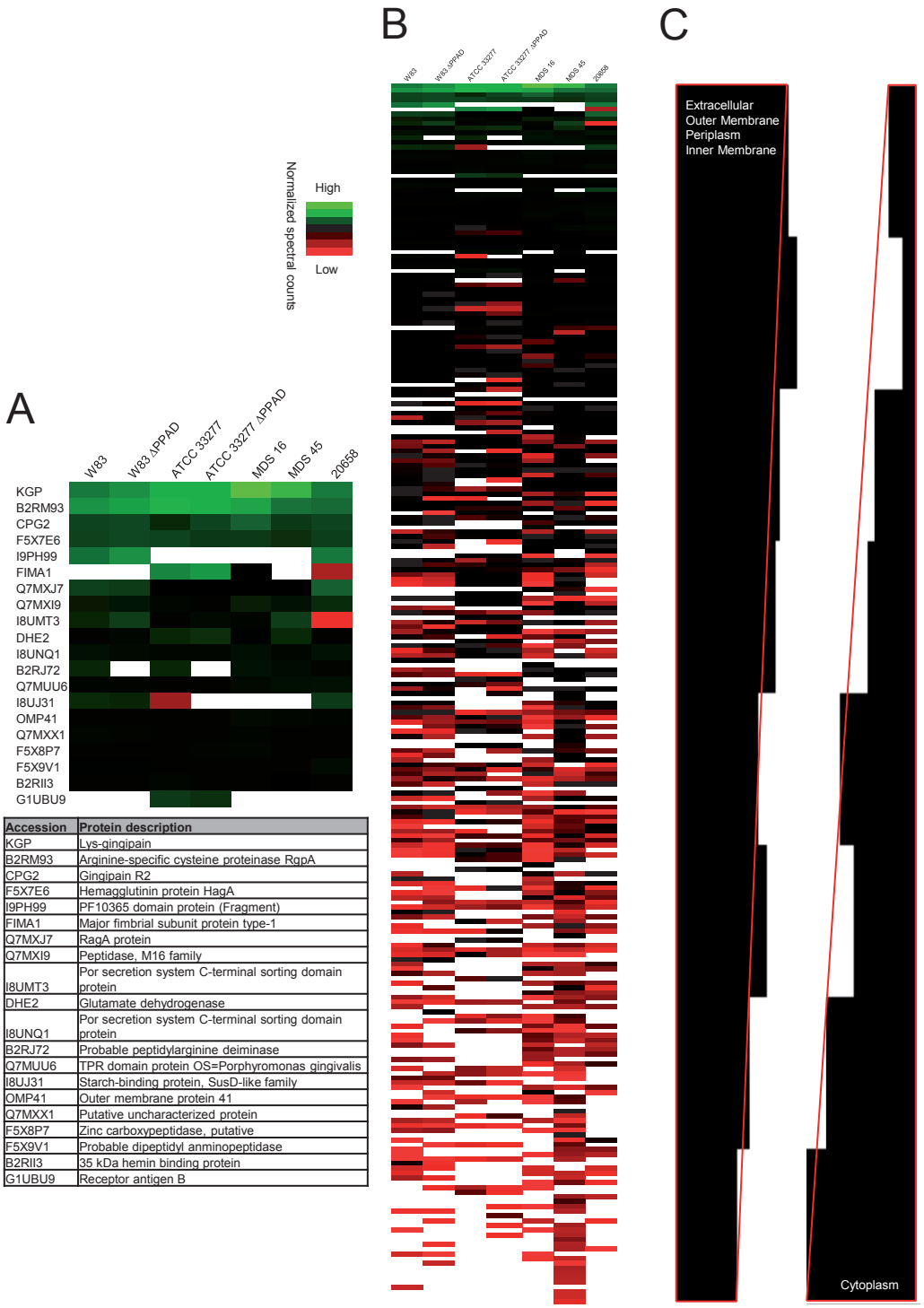


Figure 2: Localization prediction of identified proteins. The predicted subcellular or extracellular localization of the 257 identified *P. gingivalis* proteins was assessed using different algorithms, as well as a manual curation based on domain identification. Percentages of proteins for each predicted localization are shown **(A)** for the core and variable exoproteomes and **(B)** for all 257 identified proteins.

Relative protein abundance in *P. gingivalis* exoproteomes

Using normalized spectral counts, a comparison of the relative abundance of identified proteins was performed. The resulting 'protein secretion profiles' are presented in the heat maps in Figure 3. The data show that the twenty most abundant extracellular proteins were rather consistently present. Nevertheless, the relative amounts of these twenty exoproteins in the media of different strains, as reflected in spectral counts, differ statistically significantly in about 25% of the cases. Of note, the twenty most abundant exoproteins include the major known virulence factors of *P. gingivalis*, specifically the gingipains, fimbriae and PPAD (Figure 3A). High differences in relative abundance were observed for the PF10365 domain protein, the major fimbrial subunit protein type-1 (FimA), a Por secretion system C-terminal sorting domain protein, and a starch-binding protein of the SusD-like family. As expected, the PPAD protein was absent from the extracellular proteomes of the two PPAD mutants. Interestingly, the presence of different fimbriae-related serotypes was reflected in the abundance of the FimA protein. FimA was not identified in the reference strain W83, the W83 PPAD mutant and the MDS45 isolate. In contrast, FimA was found in high amounts amongst the extracellular proteins of the ATCC 33277 wild-type and PPAD mutant, and in moderate or low amounts in the MDS16 and 20658 isolates, respectively.

In contrast to the highly abundant extracellular proteins, detection of the low-abundant extracellular proteins was highly variable. This is illustrated by the heat map in Figure 3B, where the highest level of variation in detected exoproteins is observed for exoproteins of low abundance (indicated by red bars). Indeed, a statistical analysis of the differences in the amounts of the 50 least abundant exoproteins, as reflected by spectral counts, revealed that only 9% of the differences in the amounts of these exoproteins were statistically significant. This underscores the view that the detection of these low-abundance exoproteins is highly noisy. Furthermore, a clustered prediction shows that low-abundant extracellular proteins predominantly had a predicted cytoplasmic localization, while most of the highly abundant extracellular proteins were predicted to have an extracytoplasmic localization (Figure 3C).



Accession	Protein description
KGP	Lys-gingipain
B2RM93	Arginine-specific cysteine proteinase RgpA
CPG2	Gingipain R2
F5X7E6	Hemagglutinin protein HagA
I9PH99	PF10365 domain protein (Fragment)
FIMA1	Major fimbrial subunit protein type-1
Q7MXJ7	RagA protein
Q7MXI9	Peptidase, M16 family
I8UMT3	Por secretion system C-terminal sorting domain protein
DHE2	Glutamate dehydrogenase
I8UNQ1	Por secretion system C-terminal sorting domain protein
B2RJ72	Probable peptidylarginine deiminase
Q7MUU6	TPR domain protein OS=Porphyromonas gingivalis
I8UJ31	Starch-binding protein, SusD-like family
OMP41	Outer membrane protein 41
Q7MXX1	Putative uncharacterized protein
F5X8P7	Zinc carboxypeptidase, putative
F5X9V1	Probable dipeptidyl anminopeptidase
B2RII3	35 kDa hemin binding protein
G1UBU9	Receptor antigen B

Figure 3: Exoproteome profiles of the investigated *P. gingivalis* isolates. A total number of 257 extracellular proteins was identified. Relative amounts of the identified extracellular proteins are shown, based on normalized spectral counts. The y-axis represents the proteins, the x-axis shows the names of the isolates. **(A)** The twenty most abundantly secreted proteins and the respective protein descriptions. **(B)** Exoproteome profiles including the whole set of 257 identified extracellular proteins. **(C)** Clustered prediction of protein localization for the extracellular proteins displayed in panel B. The bars depict the relative abundance of proteins with predicted extracellular, outer membrane, periplasmic or inner membrane localization (left bar) *versus* the relative abundance of proteins with a predicted cytoplasmic localization (right bar) per cluster of ~32 proteins.

Predicted functions of core and variable extracellular proteins

The GO identifiers of all detected proteins were imported into REVIGO in order to visualize their functional background (Figure 4). Eight of the 64 identified proteins of the core exoproteome represented seven different biological processes based on GO terms, whereas the other 56 core proteins had unknown functions (Figure 4A). The biological processes represented by core extracellular proteins were pathogenesis, cellular amino acid metabolism, anaerobic cobalamin biosynthesis, putrescine biosynthesis, protein folding, carbohydrate metabolism and transmembrane transport. The five most abundant proteins with known functions found in the core exoproteome were the lysine-gingipain Kgp, the two arginine-gingipains RgpA and RgpB, the agglutination protein hemagglutinin A and the receptor antigen RagA.

Amongst the 193 proteins of the variable exoproteome, 72 represented 48 different biological processes based on GO terms, whereas 121 had unknown functions (Figure 4B). The most unique processes involving high numbers of the identified extracellular proteins included pathogenesis, cell redox homeostasis, protein folding, cell adhesion, iron ion transport, response to stress and biosynthesis. The five most abundant known variable proteins were the major fimbrial subunit protein type-1 (FimA), a starch-binding protein, the receptor antigen RagB, the Mfa1 fimbrilin and the minor fimbrial component FimE.

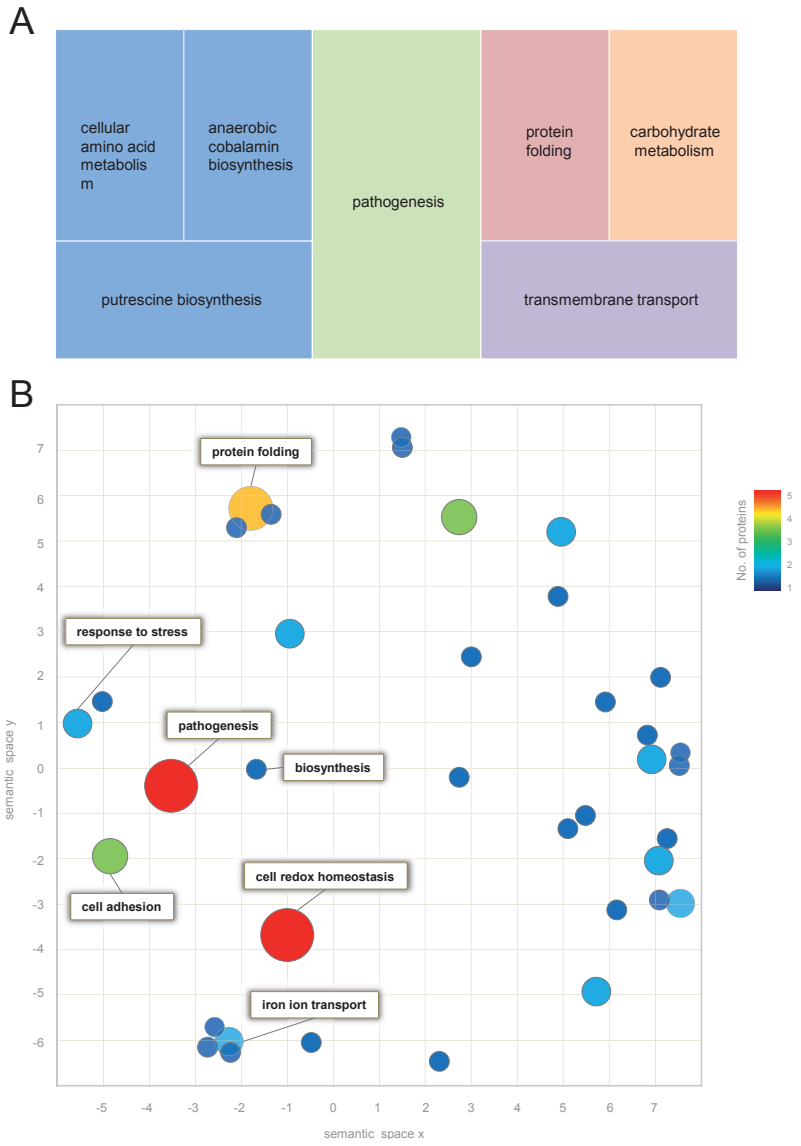


Figure 4: Functional characterization of the core and variable *P. gingivalis* exoproteome. GO terms of the present protein dataset were imported into the REVIGO software to visualize their functional background. **(A)** Tree map of the core exoproteome. Eight of the 64 identified extracellular core proteins represented seven different biological processes based on GO terms, whereas the other 56 core proteins had unknown functions. The colors indicate the different functional clusters, while the size of the rectangulars is proportional to the number of identified proteins with the respective function. **(B)** Scatter plot of the variable exoproteome. Amongst the 193 variable extracellular proteins, 72 represented 48 different biological processes based on GO terms, whereas 121 had unknown functions. The y-axis and x-axis represent the semantic space. Similar or related protein functions cluster together. The size and color of the dots represents the number of proteins identified with the respective function.

The extracellular citrullinome of *P. gingivalis*

To assess the possible citrullination of *P. gingivalis* proteins by PPAD, a search was performed for all arginine-containing peptides with according mass shifts. This resulted in a list of 25 potentially citrullinated proteins, including the gingipains, PPAD, outer membrane proteins, receptor antigens, heme-binding proteins and several uncharacterized proteins (Table 1). Of note, potential PPAD citrullination was only detectable in the reference strain W83 and the clinical isolate MDS45. Furthermore, previous studies suggested that proper processing of PPAD may be relevant for full enzymatic activity[43,44]. We therefore performed a sequence coverage analysis of the extracellular PPAD (Supplementary Figure S5). This revealed the presence of two peptides from the C-terminal domain of PPAD in the W83 reference strain and the MDS45 isolate. This C-terminal domain is usually cleaved off upon secretion, suggesting the incomplete processing of PPAD in *P. gingivalis* W83 and MDS45. In contrast, no peptides from the C-terminal domain of PPAD were detectable in the reference strain ATCC 33277 and the other clinical isolates.

Notably, the above assessment of citrullination could lead to false-positive identification of citrullinated peptides, since the same mass shift can also be caused by deamidation of asparagine and glutamine residues. To eliminate such potentially false-positive identifications, peptides containing asparagine and/or glutamine residues were excluded for a high-confidence assessment of protein citrullination. This led to the identification of six citrullinated proteins in total (Table 1), where the respective citrullinated peptides contained a C-terminal arginine residue with exception of some citrullinated peptides from RgpA which contained an internal citrulline (Supplementary Figure S6). Interestingly, the arginine-specific cysteine proteinase RgpA was only found to be citrullinated in the reference strains W83 and ATCC 33277, and the two RA-associated *P. gingivalis* isolates MDS16 and MDS45. Further citrullinated proteins were the Mfa1 fimbrilin protein (only in MDS45), as well as four uncharacterized proteins (mainly in clinical isolates). Importantly, none of these proteins was identified as being citrullinated in the PPAD-deficient mutants. Together, these data imply that the extracellular citrullinome of *P. gingivalis* consists of six to 25 proteins, including major virulence factors.

Table 1: Overview of citrullinated extracellular proteins.

A total of 25 proteins in the present dataset was identified as being tentatively citrullinated. Six of these proteins were identified as definitely citrullinated, because the respective peptides lack asparagine and glutamine residues that could potentially be deamidated. The Table shows for each protein the Uniprot accession number, protein description, molecular weight, predicted subcellular localization and biological function in GO terms. An orange square indicates the detection of one or more tentatively citrullinated tryptic peptides from a particular exoprotein of an indicated *P. gingivalis* isolate; a green square indicates the detection of one or more high-confidence citrullinated peptides; a white square indicates that no tentative or high-confidence citrullinated peptides were detected.

accession number	description	MW (kDa)	localization (pred.)	biological function	W83	W83 ΔPPAD	ATCC 33277	ATCC 33277 ΔPPAD	MDS 16	MDS 45	20658
B2RM93	Arginine-specific cysteine proteinase RgpA	185	Extracellular	pathogenesis	■	■	■	■	■	■	■
B2RLK2	Lys-gingipain	187	Extracellular	hemolysis in other organism; pathogenesis; proteolysis	■	■	■	■	■	■	■
F5XB86	Lysine-specific cysteine proteinase Kgp	188	Extracellular	unknown	■	■	■	■	■	■	■
Q7MXI9	Peptidase, M16 family	106	Cytoplasm	unknown	■	■	■	■	■	■	■
Q7MTV9	Putative uncharacterized protein	24	Outer Membrane	unknown	■	■	■		■	■	■
P95493	Gingipain R2	81	Extracellular	pathogenesis; proteolysis	■	■			■	■	■
Q9S3R9	Outer membrane protein 41	43	Outer Membrane	unknown	■	■			■	■	■
Q7MVM2	Putative uncharacterized protein	50	Outer Membrane	unknown	■	■			■	■	■
I9P773	PF14060 domain protein	31	Periplasm	unknown	■	■			■		■
Q7MT25	Putative uncharacterized protein	27	Periplasm	unknown	■				■	■	■
Q7MXJ7	RagA protein	112	Outer Membrane	transport	■	■			■		■
F5X8P7	Zinc carboxypeptidase, putative	92	Outer Membrane	unknown		■			■	■	■
B2RIM9	Heme-binding protein FetB	33	Outer Membrane	anaerobic cobalamin biosynthetic process	■				■		■
B2RJ72	Probable peptidylarginine deiminase	62	Extracellular	putrescine biosynthetic process	■					■	
Q7MUS3	Putative uncharacterized protein	96	Outer Membrane	transport					■		■
Q7MUA1	Putative uncharacterized protein	23	Periplasm	unknown						■	■
Q7MAV6	3-oxoacyl-[acyl-carrier-protein] synthase 2	45	Cytoplasm	fatty acid biosynthetic process						■	
G1UBU7	FimA type II fimbrilin	42	Outer Membrane	cell adhesion; pathogenesis						■	
B2RHG1	Mfa1fimbrilin	61	Outer Membrane	cell-cell adhesion; pathogenesis						■	
F5XAW2	Putative lipoprotein	34	Outer Membrane	unknown						■	
Q7MT41	LysM domain protein	56	Periplasm	unknown							■
B2RI00	Putative uncharacterized protein	46	Outer Membrane	unknown					■		
Q7MX91	Putative uncharacterized protein	15	Outer Membrane	unknown		■					
B2RHG7	Receptor antigen A	115	Outer Membrane	transport			■				
Q7MWY0	Tetratricopeptide repeat protein	52	Periplasm	unknown							■

Discussion

Here we present a first comparative exoproteome analysis for the oral pathogen *P. gingivalis*, including two frequently used reference strains and three clinical isolates from periodontitis patients with or without RA. Several striking observations were made. In the first place, the two widely used reference strains show a remarkable exoproteome heterogeneity, which is only partially reflected in the exoproteomes of the three clinical isolates. Secondly, the vast majority of identified extracellular proteins are predicted outer membrane proteins, followed by predicted cytoplasmic proteins. The presence and abundance of proteins belonging to the latter group was found to be highly variable. Thirdly, the functions of the identified extracellular proteins are consistent with the pathogenic lifestyle of *P. gingivalis*, which has to protect itself against severe redox stress and restricted availability of iron in the human body. Lastly, we show that six to 25 proteins of *P. gingivalis* belong to the PPAD-dependent extracellular citrullinome, including some of the major virulence factors of this pathogen.

The two reference strains W83 and ATCC 33277 are both widely studied and representative for the species of *P. gingivalis*. Nevertheless, it has been shown that both strains differ substantially in their pathogenicity in animal models[45,46]. Consistent with the later findings, our present study revealed that both strains express many different proteins in their exoproteomes. This may have impact on virulence and the potential to cause disease as was previously shown by Genco *et al.*[47]. In fact, only about a quarter of the identified exoproteins were common to all investigated *P. gingivalis* isolates, including the two reference strains. These proteins, which make up the core exoproteome, are mainly proteins predicted to be outer membrane-associated or secreted. Well-known core exoproteins are the gingipains, agglutination proteins and receptor antigens, which are all involved in virulence and host invasion[48,49]. Hence, these conserved exoproteins could be potential drug or vaccine targets.

Three quarters of the identified exoproteins were shown to be variable among the seven isolates, and included mainly proteins involved in pathogenesis, stress responses or cell adhesion. Fimbriae-related proteins were found to be the most abundant variable exoproteins. This is consistent with the fact that fimbriae are not found in all *P. gingivalis* isolates and that they show high genotypic variability. W83 for example is known as an afimbriated strain, because of the very low or absent expression of major fimbriae[50-52], which was also evidenced by the failure to detect FimA amongst the extracellular proteins of W83 in the present study. As a consequence, different *P. gingivalis* isolates vary in their capacity to form biofilms and to adhere to host cells[20,53]. Further, the observed differences in fimbriae detection could relate to differences in gene expression or the secretion and assembly of fimbrial subunits. Of note, most identified exoproteins (~35%) are predicted outer membrane proteins. This most likely relates to the fact that the outer membrane contains loosely anchored proteins, proteins that are liberated from the membrane by cleavage, and proteins that are released by the formation of outer membrane vesicles[20,54].

Intriguingly, significant differences in the total numbers of identified proteins were observed among the studied *P. gingivalis* isolates. This could either relate to differences in the activity of their secretion machinery, to differences in their susceptibility to cell lysis, or both, as previously described

for other bacteria such as *Staphylococcus aureus* [55]. Most of the additionally identified proteins in the RA patient-derived *P. gingivalis* isolate MDS45 are predicted to be cytoplasmic, which suggests that this isolate may be more susceptible to cell lysis than the other studied isolates. Besides MDS45, also the reference strain ATCC 33277 and the corresponding PPAD mutant were found to contain a relatively high proportion of predicted cytoplasmic proteins in their growth medium. Although it remains to be shown whether the predicted cytoplasmic proteins that were found extracellularly are actively secreted or released by cell lysis, these proteins may interact with the human host. For example, they could impact on the immune system by exposing new epitopes which, conceivably, might lead to the production of (auto-)antibodies in a person genetically prone to develop RA. Besides that, surface-exposed cytoplasmic proteins could have particular functions related to virulence, including iron metabolism, immune evasion and the adherence to host tissues[56-59]. Compared to other bacteria, like *S. aureus*, Group A streptococci and *Mycobacterium tuberculosis*[60,61], whose exoproteomes can contain more than 50% predicted cytoplasmic proteins, *P. gingivalis* seems to be a less "leaky" bacterium with a more intact membrane integrity, although this may relate to the particular growth conditions applied. It should be noted that extracellular proteases, such as the gingipains, may also impact on the number of cytoplasmic proteins detected in the growth medium. As recently shown for *Bacillus subtilis*, extracellular proteases may degrade cytoplasmic proteins upon cell lysis and, additionally, they may degrade particular autolysins, which would lead to reduced cell lysis[62-64]. In both scenarios, the result is a lower amount of detectable cytoplasmic proteins in the growth medium. Consistent with this view, the *P. gingivalis* isolate MDS16, which showed the highest gingipain levels, displayed the lowest proportion of predicted cytoplasmic proteins in the growth medium.

The extracellular presence of PPAD is a common trait of all investigated wild-type *P. gingivalis* isolates[17], and could be a possible connection between *P. gingivalis* and RA[11,13]. Therefore, the extracellular citrullinome of *P. gingivalis* was analysed. To this end, citrullinated and/or deamidated arginine-containing peptides were first identified. To eliminate false-positive identifications, potentially deamidated peptides were excluded from the analysis in a second step. Twenty-five proteins were found to match the first criterion, and these were shown to be mainly extracellular and membrane-associated proteins, such as proteinases, peptidases or porins. These proteins are probably also exposed to the outer surface in an *in vivo* situation and might thus increase the total citrullination burden in the human host. The list of tentatively citrullinated proteins includes the PPAD of the W83 and MDS45 isolates, but not the PPAD of the other investigated isolates. Intriguingly, the tentative citrullination of PPAD in the W83 and MDS45 isolates correlates with the detection of the full-length version of this enzyme, and seems to be lost upon cleavage of the C-terminal domain. In this context it is noteworthy that Quirke and colleagues reported that recombinant full-length PPAD produced in *Escherichia coli* becomes auto-citrullinated, while Konig and colleagues suggested that this auto-citrullination could be an artefact of the cloning procedure that might not occur in *P. gingivalis* due to N-terminal cleavage of the protein[43,65]. Further, by excluding potentially deamidated peptides, a set of six proteins was identified as definitely citrullinated. These include the gingipain RgpA produced by the reference strains and the isolates from RA patients, but not the RgpA from isolate 20658, which was

obtained from a periodontitis patient. This raises the question whether citrullination of RgpA could be a factor involved in the development of RA, especially since this citrullinated protein is secreted in high amounts. In this respect it is noteworthy that Kharlamova *et al.* recently showed that antibodies against another gingipain, RgpB, positively correlated with periodontitis, RA and ACPA-positivity[66]. However, a possible correlation with antibodies against RgpA has not been assessed yet and should be subject of future investigations. Besides RgpA, four unknown proteins and the fimbrial protein Mfa1 were found to be citrullinated. Citrullination of surface structures, such as the fimbriae, could also represent a potential trigger of ACPA formation, since these structures are among the first to make contact with immune cells of the host (e.g. macrophages or dendritic cells)[67]. Conceivably, this could trigger an autoimmune response against these and other citrullinated proteins in the host. Investigation of the characteristics of the identified citrullinated peptides revealed that most of these peptides contained a C-terminal citrulline, except for some peptides of RgpA that contained an internal citrulline (Figure S6). A recent study by Bennike *et al.* showed that trypsin is unable to cleave C-terminally of citrulline residues, which can be used to verify the citrullination of particular peptides through a lack of trypsin-mediated cleavage of these peptides[68]. Furthermore, Bennike *et al*. concluded that manual curation of tentative citrullinated peptides is essential. Another study by Wegner *et al.* indicated that PPAD is only able to citrullinate C-terminal arginine residues, which implies that proteins first need to be cleaved by the arginine-specific gingipains RgpA or RgpB to expose a C-terminal arginine that can then serve as substrate for PPAD[18]. Indeed, the peptides presently identified as being citrullinated mostly contained C-terminal citrulline residues and, in fact, many of them represented the C-termini of the respective proteins (Figure S6). Hence, these peptides were probably citrullinated by PPAD while being part of the native protein. Other identified peptides with a C-terminal citrulline, which are located within the polypeptide chain of particular proteins, were probably first processed by RgpA or RgpB and then citrullinated by PPAD, as was proposed by Wegner *et al.* The few peptides with tentative internal citrulline residues may either represent mis-annotations, or could reflect the presumably infrequent citrullination of internal arginine residues by PPAD.

Conclusion

In conclusion, the present study provides a broad overview on the heterogeneity of the extracellular proteome and citrullinome of the important oral pathogen *P. gingivalis*. Main differences were found in the extracellular presence of low-abundant predicted cytoplasmic proteins and in the citrullination status of particular proteins, while the presence of most of the known highly abundant virulence factors was demonstrated for all investigated isolates. With respect to the development of RA, our observations focus special attention on the six to 25 proteins that were found to be (potentially) citrullinated, especially the gingipain RgpA. Accordingly, our future studies will include larger collections of *P. gingivalis* isolates, and they will focus attention on possible correlations between particular *P. gingivalis* exoproteome or citrullinome profiles and the inflammatory diseases periodontitis and RA.

Supporting Information

Table S1 – Overview of identified extracellular proteins in the present study with peptide and spectral count information
Figure S1 – Growth curves of *P. gingivalis* in liquid culture
Figure S2 – LDS-PAGE of exoproteome samples
Figure S3 – Numbers of proteins in the exoproteomes of the *P. gingivalis* isolates
Figure S4 – Localization prediction of identified proteins per isolate
Figure S5 – Sequence coverage and modifications of PPAD
Figure S6 – Spectra and fragmentation tables of citrullinated proteins
Supplementary FASTA file – Non-redundant *P. gingivalis* database concatenated with common laboratory contaminants and a target-pseudoreversed decoy database

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References

1. Jansson, H. Studies on periodontitis and analyses of individuals at risk for periodontal diseases. *Swed. Dent. J. Suppl.* 5–49 (2006).
2. Berthelot, J.-M. & Le Goff, B. Rheumatoid arthritis and periodontal disease. *Jt., Bone, Spine* 77, 537–41 (2010).
3. Yilmaz, O. The chronicles of *Porphyromonas gingivalis*: the microbium, the human oral epithelium and their interplay. *Microbiology (Reading, Engl.)* 154, 2897–903 (2008).
4. Bostanci, N. & Belibasakis, G. N. *Porphyromonas gingivalis*: an invasive and evasive opportunistic oral pathogen. *FEMS Microbiol. Lett.* 333**,** 1–9 (2012).
5. Griffen, A. L., Becker, M. R., Lyons, S. R., Moeschberger, M. L. & Leys, E. J. Prevalence of *Porphyromonas gingivalis* and periodontal health status. *J. Clin. Microbiol.* 36, 3239–42 (1998).
6. van Winkelhoff, A. J., Loos, B. G., van der Reijden, W. A. & van der Velden, U. *Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. *J. Clin. Periodontol.* 29, 1023–8 (2002).
7. Lundberg, K., Wegner, N., Yucel-Lindberg, T. & Venables, P. J. Periodontitis in RA—the citrullinated enolase connection. *Nat. Rev. Rheumatol.* 6, 727–730 (2010).
8. Hajishengallis, G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* 15, 30–44 (2014).
9. de Smit, M. *et al.* Periodontitis in established rheumatoid arthritis patients: a cross-sectional clinical, microbiological and serological study. *Arthritis Res. Ther.* 14, R222 (2012).
10. Routsias, J. G., Goules, J. D., Goules, A., Charalampakis, G. & Pikazis, D. Autopathogenic correlation of periodontitis and rheumatoid arthritis. *Rheumatology (Oxford)* 50, 1189–93 (2011).
11. de Smit, M. J., Brouwer, E., Vissink, A. & van Winkelhoff, A. J. Rheumatoid arthritis and periodontitis; a possible link via citrullination. *Anaerobe* 17, 196–200 (2011).
12. van der Helm-van Mil, A. H. M. *et al.* Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis Res. Ther.* 7, R949–58 (2005).
13. Mangat, P., Wegner, N., Venables, P. J. & Potempa, J. Bacterial and human peptidylarginine deiminases: targets for inhibiting the autoimmune response in rheumatoid arthritis? *Arthritis Res. Ther.* 12, 209 (2010).
14. Mohamed, B. M. *et al.* Citrullination of proteins: a common post-translational modification pathway induced by different nanoparticles *in vitro* and *in vivo*. *Nanomedicine* 7, 1181–95–1195 (2012).
15. Thompson, P. R. & Fast, W. Histone citrullination by protein arginine deiminase: is arginine methylation a green light or a roadblock? *ACS Chem. Biol.* 1, 433–41 (2006).
16. Baka, Z. *et al.* Citrullination under physiological and pathological conditions. *Jt., Bone, Spine* 79, 431–6 (2012).
17. Gabarrini, G. *et al.* The peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis*. *Sci. Rep.* 5, 13936 (2015).
18. Wegner, N. *et al.* Peptidylarginine deiminase from *Porphyromonas gingivalis* citrullinates human fibrinogen and α-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum.* 62, 2662–72 (2010).
19. McGraw, W. T., Potempa, J., Farley, D. & Travis, J. Purification, characterization, and sequence analysis of a potential virulence factor from *Porphyromonas gingivalis*, peptidylarginine deiminase. *Infect. Immun.* 67, 3248–56 (1999).
20. Yoshimura, F., Murakami, Y., Nishikawa, K., Hasegawa, Y. & Kawaminami, S. Surface components of *Porphyromonas gingivalis*. *J. Periodont. Res.* 44, 1–12 (2008).
21. Yoshimura, M. *et al.* Proteome analysis of *Porphyromonas gingivalis* cells placed in a subcutaneous chamber of mice. *Oral Microbiol. Immunol.* 23, 413–8 (2008).
22. Osbourne, D. *et al.* VimA-dependent modulation of the secretome in *Porphyromonas gingivalis*. *Mol. Oral. Microbiol.* 27, 420–35 (2012).

23. Hendrickson, E. L., Xia, Q., Wang, T., Lamont, R. J. & Hackett, M. Pathway analysis for intracellular *Porphyromonas gingivalis* using a strain ATCC 33277 specific database. *BMC Microbiol.* 9, 185 (2009).

24. Zhang, Y. *et al.* Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components. *Proteomics* 5, 198–211 (2004).

25. Cogo, K. *et al.* Proteomic analysis of *Porphyromonas gingivalis* exposed to nicotine and cotinine. *J. Periodont. Res.* 47, 766–75 (2012).

26. Xia, Q. *et al.* Quantitative proteomics of intracellular *Porphyromonas gingivalis*. *Proteomics* 7, 4323–37 (2007).

27. Maeda, K., Nagata, H., Ojima, M. & Amano, A. Proteomic and Transcriptional Analysis of Interaction between Oral Microbiota *Porphyromonas gingivalis* and *Streptococcus oralis*. *J. Proteome Res.* 14, 82–94 (2014).

28. Veloo, A. C. M., Elgersma, P. E., Friedrich, A. W., Nagy, E. & van Winkelhoff, A. J. The influence of incubation time, sample preparation and exposure to oxygen on the quality of the MALDI-TOF MS spectrum of anaerobic bacteria. *Clin. Microbiol. Infect.* 20, O1091–7 (2014).

29. Dreisbach, A. *et al.* Profiling the surfacome of *Staphylococcus aureus*. *Proteomics* 10, 3082–96 (2010).

30. Bonn, F. *et al.* Picking vanished proteins from the void: how to collect and ship/share extremely dilute proteins in a reproducible and highly efficient manner. *Anal. Chem.* 86, 7421–7 (2014).

31. Vizcaíno, J. A. *et al.* ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* 32, 223–226 (2014).

32. Krogh, A. *et al.* Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci.* 12, 1652 (2003).

33. Berven, F. S. *et al.* Analysing the outer membrane subproteome of *Methylococcus capsulatus* (Bath) using proteomics and novel biocomputing tools. *Arch. Microbiol.* 184, 362–77 (2005).

34. Sonnhammer, E. L., Heijne, von, G. & Krogh, A. A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 6, 175–82 (1998).

35. Krogh, A., Larsson, B., Heijne, von, G. & Sonnhammer, E. L. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–80 (2001).

36. Käll, L., Krogh, A. & Sonnhammer, E. L. L. A combined transmembrane topology and signal peptide prediction method. *J. Mol. Biol.* 338, 1027–36 (2004).

37. Petersen, T. N., Brunak, S., Heijne, von, G. & Nielsen, H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–6 (2011).

38. Hiller, K., Grote, A., Scheer, M., Münch, R. & Jahn, D. PrediSi: prediction of signal peptides and their cleavage positions. *Nucleic Acids Res.* 32, W375–9 (2004).

39. Bendtsen, J. D., Kiemer, L., Fausbøll, A. & Brunak, S. Non-classical protein secretion in bacteria. *BMC Microbiol.* 5, 58 (2005).

40. Yu, N. Y. *et al.* PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26, 1608–15 (2010).

41. Paramasivam, N. & Linke, D. ClubSub-P: Cluster-Based Subcellular Localization Prediction for Gram-Negative Bacteria and Archaea. *Front. Microbiol.* 2, 218 (2011).

42. Supek, F., Bošnjak, M., Škunca, N. & Šmuc, T. REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS ONE* 6, e21800 (2011).

43. Konig, M. F., Paracha, A. S., Moni, M., Bingham, C. O. & Andrade, F. Defining the role of *Porphyromonas gingivalis* peptidylarginine deiminase (PPAD) in rheumatoid arthritis through the study of PPAD biology. *Ann. Rheum. Dis.* 74, 2054–61 (2014).

44. Rodríguez, S. B., Stitt, B. L. & Ash, D. E. Expression of peptidylarginine deiminase from *Porphyromonas gingivalis* in *Escherichia coli*: enzyme purification and characterization. *Arch. Biochem. Biophys.* 488, 14–22 (2009).

45. Neiders, M. E. *et al.* Heterogeneity of virulence among strains of *Bacteroides gingivalis*. 24, 192–198 (1989).

46. Igboin, C. O., Moeschberger, M. L., Griffen, A. L. & Leys, E. J. *Porphyromonas gingivalis* virulence in a *Drosophila melanogaster* model. *Infect. Immun.* 79, 439–448 (2010).

47. Genco, C. A. *et al.* A novel mouse model to study the virulence of and host response to *Porphyromonas* (*Bacteroides*) gingivalis. *Infect. Immun.* 59, 1255–1263 (1991).

48. Chen, T., Nakayama, K., Belliveau, L. & Duncan, M. J. *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. *Infect. Immun.* 69, 3048–3056 (2001).

49. Curtis, M. A. *et al.* Attenuation of the virulence of *Porphyromonas gingivalis* by using a specific synthetic Kgp protease inhibitor. *Infect. Immun.* 70, 6968–6975 (2002).

50. Zheng, C., Wu, J. & Xie, H. Differential expression and adherence of *Porphyromonas gingivalis* FimA genotypes. *Mol. Oral. Microbiol.* 26, 388–395 (2011).

51. Hayashi, J., Nishikawa, K., Hirano, R., Noguchi, T. & Yoshimura, F. Identification of a two-component signal transduction system involved in fimbriation of *Porphyromonas gingivalis*. *Microbiol. Immunol.* 44, 279–282 (2000).

52. Nishikawa, K. & Duncan, M. J. Histidine kinase-mediated production and autoassembly of *Porphyromonas gingivalis* fimbriae. *J. Bacteriol.* 192**,** 1975–1987 (2010).

53. Barbosa, G. M., Colombo, A. V., Rodrigues, P. H. & Simionato, M. R. L. Intraspecies Variability Affects Heterotypic Biofilms of *Porphyromonas gingivalis* and *Prevotella intermedia*: Evidences of Strain-Dependence Biofilm Modulation by Physical Contact and by Released Soluble Factors. *PLoS ONE* 10, e0138687 (2015).

54. Veith, P. D. *et al. Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J. Proteome Res.* 13**,** 2420–2432 (2014).

55. Matsuda, K., Nakamura, K., Adachi, Y., Inoue, M. & Kawakami, M. Autolysis of methicillin-resistant *Staphylococcus aureus* is involved in synergism between imipenem and cefotiam. *Antimicrob. Agents Chemother.* 39, 2631–2634 (1995).

56. Mohan, S. *et al.* Tuf of *Streptococcus pneumoniae* is a surface displayed human complement regulator binding protein. *Mol. Immunol.* 62**,** 249–264 (2014).

57. Boradia, V. M., Raje, M. & Raje, C. I. Protein moonlighting in iron metabolism: glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Biochem. Soc. Trans.* 42, 1796–1801 (2014).

58. Dreisbach, A., van Dijl, J. M. & Buist, G. The cell surface proteome of *Staphylococcus aureus*. *Proteomics* 11, 3154–3168 (2011).

59. Henderson, B. An overview of protein moonlighting in bacterial infection. *Biochem. Soc. Trans.* 42, 1720–1727 (2014).

60. Hempel, K., Herbst, F.-A., Moche, M., Hecker, M. & Becher, D. Quantitative proteomic view on secreted, cell surface-associated, and cytoplasmic proteins of the methicillin-resistant human pathogen *Staphylococcus aureus* under iron-limited conditions. *J. Proteome Res.* 10**,** 1657–66 (2011).

61. Tjalsma, H. *et al.* Proteomics of protein secretion by *Bacillus subtilis*: separating the 'secrets' of the secretome. *Microbiol. Mol. Biol. Rev.* 68, 207–233 (2004).

62. Antelmann, H. *et al.* A proteomic view on genome-based signal peptide predictions. *Genome Res.* 11, 1484–1502 (2001).

63. Krishnappa, L., Monteferrante, C. G., Neef, J., Dreisbach, A. & van Dijl, J. M. Degradation of extracytoplasmic catalysts for protein folding in *Bacillus subtilis*. *Appl. Environ. Microbiol.* 80, 1463–1468 (2013).

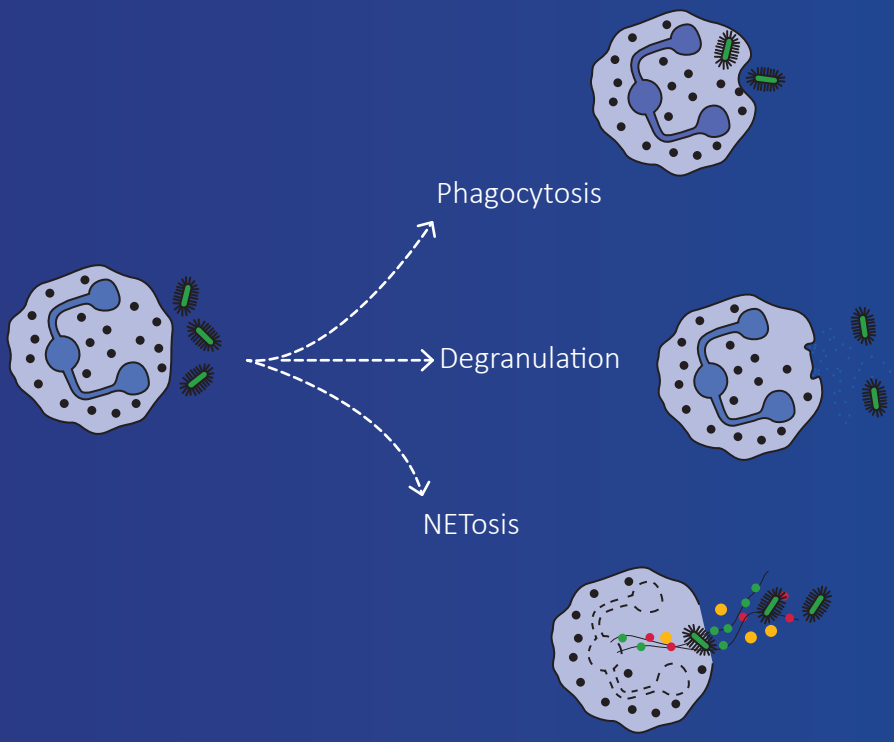
64. Krishnappa, L. *et al.* Extracytoplasmic proteases determining the cleavage and release of secreted proteins, lipoproteins, and membrane proteins in *Bacillus subtilis*. *J. Proteome Res.* 12, 4101–4110 (2013).

65. Quirke, A.-M. *et al.* Heightened immune response to autocitrullinated *Porphyromonas gingivalis* peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis. *Ann. Rheum. Dis.* 73, 263–269 (2014).

66. Kharlamova, N. *et al.* Antibodies to *Porphyromonas gingivalis* Indicate Interaction Between Oral Infection, Smoking, and Risk Genes in Rheumatoid Arthritis Etiology. *Arthritis Rheumatol* 68, 604–613 (2016).

67. Takeshita, A. *et al. Porphyromonas gingivalis* fimbriae use beta2 integrin (CD11/CD18) on mouse peritoneal macrophages as a cellular receptor, and the CD18 beta chain plays a functional role in fimbrial signaling. *Infect. Immun.* 66, 4056–4060 (1998).

68. Bennike, T. *et al.* Optimizing the Identification of Citrullinated Peptides by Mass Spectrometry: Utilizing the Inability of Trypsin to Cleave after Citrullinated Amino Acids. *J. Proteomics Bioinform.* 6, 1–8 (2013).


Phagocytosis
Degranulation
NETosis

CHAPTER 5

A secreted bacterial peptidylarginine deiminase can neutralize human innate immune defenses

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Abstract

The keystone oral pathogen *Porphyromonas gingivalis* is associated with severe periodontitis. Intriguingly, this bacterium is known to secrete high amounts of an enzyme that converts peptidylarginine into citrulline residues. The present study was aimed at identifying possible functions of this citrullinating enzyme, named '*Porphyromonas* peptidylarginine deiminase' (PPAD), in the periodontal environment. The results show that PPAD is detectable in the gingiva of patients with periodontitis, and that it literally '*neutralizes*' human innate immune defenses at three distinct levels, namely bacterial phagocytosis, capture in neutrophil extracellular traps (NETs), and killing by the lysozyme-derived cationic antimicrobial peptide LP9. As shown by mass spectrometry, exposure of neutrophils to PPAD-proficient bacteria reduces the levels of neutrophil proteins involved in phagocytosis and the bactericidal histone H2. Further, PPAD is shown to citrullinate the histone H3, thereby facilitating the bacterial escape from NETs. Lastly, PPAD is shown to citrullinate LP9, thereby restricting its antimicrobial activity. The importance of PPAD for immune evasion is corroborated in the infection model *Galleria mellonella*, which only possesses an innate immune system. Together, the present observations show that PPAD-catalyzed protein citrullination defuses innate immune responses in the oral cavity, and that the citrullinating enzyme of *P. gingivalis* represents a new type of bacterial immune evasion factor.

Importance

Bacterial pathogens do not only succeed in breaking the barriers that protect humans from infection, but they also manage to evade insults from the human immune system. The importance of the present study resides in the fact that protein citrullination is shown to represent a new bacterial mechanism for immune evasion. In particular, the oral pathogen *P. gingivalis* employs this mechanism to defuse innate immune responses by secreting a protein-citrullinating enzyme. Of note, this finding impacts not only on the global health problem of periodontitis, but it also extends to the prevalent autoimmune disease rheumatoid arthritis, which has been strongly associated with periodontitis, PPAD activity and loss of tolerance against citrullinated proteins such as the histone H3.

Introduction

Periodontitis affects around 10 to 15% of the adult population making it one of the most prevalent diseases worldwide[1]. It is characterized by chronic inflammation of the tissues supporting the teeth and is associated with a dysbiotic oral microbiome found primarily in the form of biofilms in the periodontal pocket (Fig. 1a). These conditions trigger an increased tissue infiltration by immune cells, mainly neutrophils, which play a pivotal role in maintaining periodontal health by employing diverse and potent bactericidal mechanisms[2,3]. Successful periodontal pathogens, however, have evolved sophisticated strategies to avoid or subvert neutrophil killing and to thrive in an inflamed environment. In particular, the Gram-negative anaerobe *Porphyromonas gingivalis*, which is considered a major etiological agent of periodontitis, possesses the ability to dysregulate the homeostasis between oral biofilms and innate immunity[2,3]. The bacterium secretes high amounts of a unique enzyme, the *P. gingivalis* peptidylarginine deiminase (PPAD), which catalyzes citrullination of both bacterial and host proteins[4-8]. This post-translational protein modification involves the deimination of positively-charged arginine residues into neutral citrulline residues. Intriguingly, *P. gingivalis* has not only been implicated in periodontitis, but also in the prevalent autoimmune disease rheumatoid arthritis, which is strongly associated with periodontitis, PPAD activity and the loss of tolerance against citrullinated proteins such as the histone H3[2,9-11]. Nonetheless, the biological and clinical relevance of PPAD for dysbiosis in the oral cavity had so far remained enigmatic. The question raised in our present study was whether this citrullinating enzyme may literally neutralize human innate immune defenses in the periodontal environment thereby serving as a secreted bacterial immune evasion factor.

Results and Discussion

PPAD impairs bacterial binding and internalization by neutrophils

To verify the relevance of PPAD production in inflamed periodontal tissue, we performed immunohistochemistry using a previously developed PPAD-specific antibody. As shown in Figure 1b, this allowed us to detect the presence of PPAD in gingival tissues of periodontitis patients for the first time. This observation enticed us to further investigate the interaction of *P. gingivalis* with key host immune cells. In particular, we aimed this investigation at dissecting potentially pleiotropic functions of PPAD in the evasion of neutrophil-specific innate immunity by *P. gingivalis* W83, previously characterized as one of the most virulent *Porphyromonas* strains[12]. Challenge with human neutrophils showed that strain W83 is bound and internalized by these neutrophils (Fig. 2a, b). Notably, the association and internalization levels observed for a genetically engineered PPAD-deficient *P. gingivalis* mutant were two- to three-fold higher compared to the parental W83 strain (Fig. 2b). This is partly related to a higher percentage of the neutrophils binding and internalizing PPAD-deficient *P. gingivalis* (Fig.S1a). Addition of PPAD-containing culture supernatant allowed the PPAD-deficient mutant to evade neutrophil association and internalization, and significant evasion of neutrophil internalization was even observed upon addition

of purified recombinant PPAD (Fig. 2c, d). This shows that PPAD helps *P. gingivalis* to evade destruction by neutrophils, which is a prerequisite to survive the high neutrophil influx in inflamed gingival tissue of periodontitis patients.

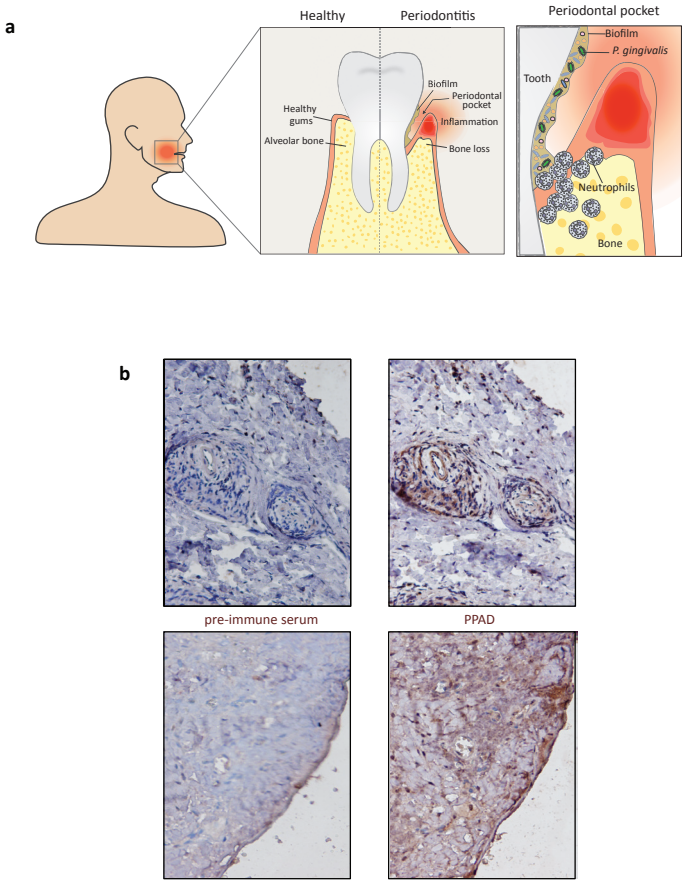


Figure 1: **Detection of PPAD in gingival tissue of a periodontitis patient. a,** Hallmarks of periodontitis: schematic representation of biofilm formation and neutrophil recruitment in the periodontal pocket. Note that the periodontal biofilm is polymicrobial, where *P. gingivalis* is represented in green and other microorganisms in orange and blue. **b**, PPAD detection by immunohistochemistry in gingival tissues of a periodontitis patient using a PPAD-specific antibody. Control staining of the same gingival tissues was performed with the respective rabbit pre-immune serum. PPAD staining is observed in gingival tissue primarily around blood vessels (upper panels) or at the epithelium (lower panels).

We have recently shown that PPAD is secreted in two different forms, either in a soluble state or bound to excreted outer membrane vesicles (OMVs)[7,8]. As shown with the recombinant protein, soluble PPAD can limit neutrophil internalization, and the same effect was observed upon addition of purified PPAD-containing OMVs to the PPAD-deficient *P. gingivalis* (Fig. 2d, Fig. S1b, c). Moreover, these OMVs even inhibited binding of the PPAD mutant bacteria by neutrophils (Fig. 2c). Together, these observations imply that both forms of secreted PPAD, soluble and OMV-bound, can serve to protect *P. gingivalis* against containment and elimination by human neutrophils. Further, the data suggest that OMV-bound PPAD could be primarily used by *P. gingivalis* to evade neutrophil-binding while the soluble PPAD might be more effective against internalization. With respect to the latter idea, it is important to bear in mind that the recombinant PPAD isolated from *Lactococcus lactis*, though soluble and enzymatically active, may lack particular as yet unidentified post-translational modifications that are present in the soluble PPAD produced by *P. gingivalis*. Such modifications could impact, for example, on the enzyme's substrate specificity and specific activity. This awaits further experimental verification by purification of soluble PPAD from the *P. gingivalis* W83 growth medium and subsequent functional and structural characterization.

How could PPAD mediate neutrophil evasion? An attractive hypothesis is that this involves the so-called gingipains of *P. gingivalis*, a group of highly proteolytic enzymes including the arginine-specific enzymes RgpA and RgpB[13,14]. We recently reported that these gingipains are subject to citrullination by PPAD[6]. Further, Maekawa *et al.* have previously shown that RgpA and RgpB induce a TLR2-C5aR crosstalk, ultimately leading to the inhibition of actin polymerization and consequently inhibition of phagocytosis. We therefore assessed the RgpA and RgpB levels by Western blotting. As shown in Figures 3a and S1d, the neutrophils are exposed to lower levels of RgpA and RgpB in the absence of PPAD. Moreover, the overall proteolytic activity in the growth medium of PPAD-deficient *P. gingivalis* is significantly reduced, as shown by a lowered rate of histone H3 protein degradation by PPAD-deficient W83 compared to the PPAD-proficient strain (Fig. 3b). Overall, in accordance with the model of Maekawa and colleagues, a lower level of RgpA and RgbB at the neutrophil surface, as observed for neutrophils infected with PPAD-deficient bacteria, will lead to less suppression of phagocytosis and therefore enhanced internalization of these bacteria as shown in Figure 2b. The underlying mechanism by which the presence of PPAD results in increased levels and activity of RgpA and RgpB is likely to be their previously documented citrullination by PPAD[6], which could confer protection against possible (self-)cleavage at arginine residues.

Furthermore, to verify the possibility that phagocytosis in neutrophils is decreased due to lower actin polymerization in the presence of PPAD-proficient bacteria, we applied a mass spectrometry-based approach. Indeed, the results show that the levels of the actin assembly-related proteins dynamin-2[15], actin-related protein 2/3[16] and the cell division control protein 42[17] are decreased when neutrophils are challenged by wild-type *P. gingivalis* (Fig. 3d). This is consistent with a role of gingipain citrullination in the inhibition of actin polymerization and evasion of phagocytosis. However, our mass spectrometry analyses provide more clues how *P. gingivalis* corrupts the neutrophil. For example, the immunoglobulin κ constant protein is not detectable in neutrophils infected with wild-type *P. gingivalis*, while this protein is identified in neutrophils infected with the PPAD mutant (Fig. 3d). This implies a role of PPAD in

inhibiting opsonization of the bacteria as immunoglobulins are important in opsonization, which is the first step of phagocytosis.

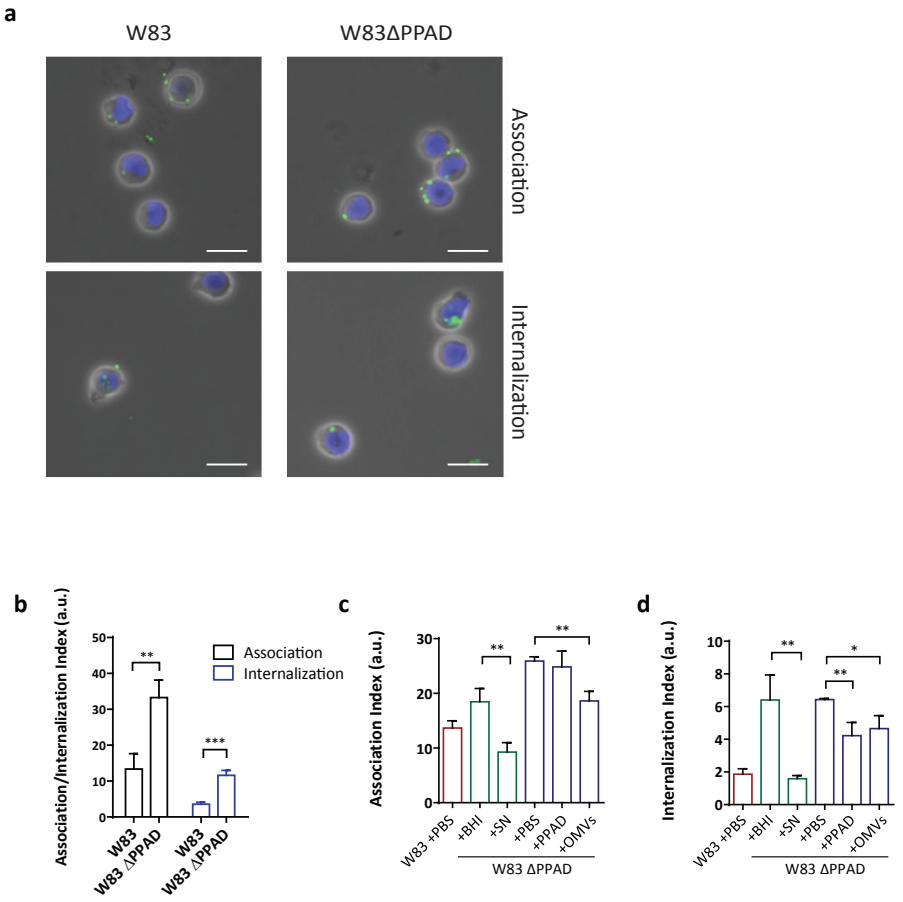
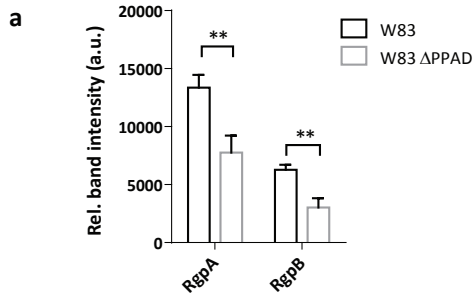
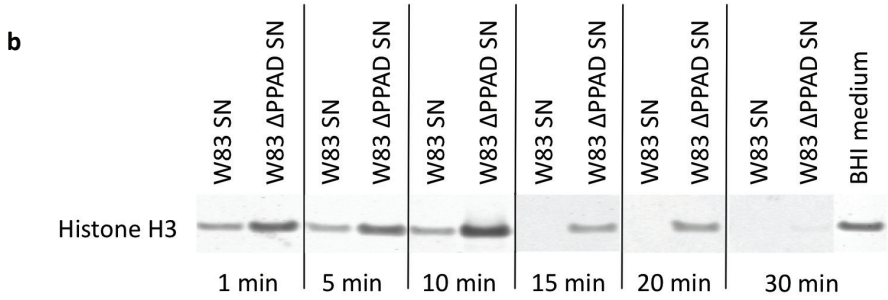


Figure 2: PPAD impairs bacterial binding and internalization by neutrophils. a, b, *P. gingivalis* W83 ΔPPAD is bound and internalized by neutrophils at a higher rate than wild-type *P. gingivalis* W83. Microscopic visualization of neutrophils with bound or internalized *P. gingivalis* (**a;** scale bars 10 µm), and the respective association and internalization indices as determined by flow cytometry (**b**). **c-d,** Rescue of bacterial binding and internalization by neutrophils upon addition of 2.5 µg recombinant PPAD (indicated as PPAD), 16 µg PPAD-containing W83 outer membrane vesicles (OMVs) or 100 µL PPAD-containing W83 culture supernatant (SN). Association and internalization indices determined by flow cytometry are shown. Data in (**b**) are means of three biologically independent samples (neutrophils from three donors), where each infection experiment was carried out four times. Data in (**c**) and (**d**) are means of four replicates of one biological sample (one neutrophil donor). **P*<0.05, ***P*<0.01, ****P*<0.001, two-tailed unpaired Student's *t*-tests. Data are presented as mean values ±SD.





c

Protein Name	Impact of PPAD	Normalized Spectral Counts	
		Neutrophils + W83	Neutrophils + W83 ΔPPAD
Dynamin-2	↓	not identified	3.86
Immunoglobulin κ constant	↓	not identified	2.26
Neutrophil cytosol factor 4	↓	not identified	2.35
Sorting nexin-3	↓	not identified	1.67
Actin-related protein 2/3 complex subunit 3	↓*	2.43	5.84
Cell division control protein 42 homolog	↓*	5.34	9.38
Integrin α-M	↓*	33.59	40.66
Integrin β-2	↓*	32.01	45.55
Annexin A3	↑	4.67	not identified

d

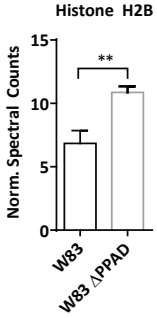


Figure 3: **PPAD stabilizes gingipains and modulates the levels of phagocytosis-related proteins. a**, Relative levels of gingipains (RgpA/RgpB) in infected neutrophils. **b,** Time course of histone H3 degradation by *P. gingivalis* proteases in the presence or absence of PPAD as determined by Western blot (SN, culture supernatant)**. c, d**, Quantification of significant changes in the amounts of the antimicrobial histone H2b (**c**) and phagocytosis-related neutrophil proteins (**d**) in infected neutrophils as approximated by mass spectrometry. Data in (**a** and **c**) are means of three replicates of one neutrophil donor. **P<0.01, two-tailed unpaired Student's *t*-tests. Data are presented as mean values ±SD. Data in (**c**) are means of three replicates of one neutrophil donor. *P<0.05, Fisher's exact test. Green and red arrows indicate up- or down-regulation of >10% of the respective protein in W83-infected neutrophils.

Altogether, a challenge with wild-type *P. gingivalis* leads to altered levels of 17 phagocytosis-related proteins compared to the PPAD mutant (Table S1). In particular, the levels of the integrins α-M and β-2, involved in actin polymerization[18], are reduced (Fig. 3d). These integrins play also crucial roles in cell signalling, neutrophil adhesion to endothelial cells, and granule exocytosis for releasing bactericidal toxins into the intracellular milieu[19]. In fact, once a bacterial prey is internalized by neutrophils, several granule and cytosolic proteins facilitate its efficient destruction. Among these, the neutrophil cytosolic factor 4 (NCF4/p40phox) is involved in the oxidative burst that serves to kill internalized bacteria[20]. Indeed, the NCF4 levels are also substantially lower when neutrophils are challenged with wild-type *P. gingivalis* compared to PPAD-deficient bacteria (Fig. 3c). Lastly, the bactericidal histone H2B[21] is present in lower amounts when neutrophils are exposed to PPAD-proficient *P. gingivalis* (Fig. 3d). Altogether, these findings show that *P. gingivalis* needs PPAD to escape internalization and subsequent elimination by neutrophils. Further, our results correlate the increased phagocytosis in the absence of PPAD to reduced levels of gingipains and a restricted impact of PPAD-deficient *P. gingivalis* on neutrophil proteins needed for phagocytosis.

PPAD citrullinates histone H3 and helps to evade neutrophil extracellular traps (NETs)

Neutrophils can also capture bacteria with NETs, which are web-like structures mainly consisting of de-condensed chromatin and bactericidal proteins[22,23]. Recent studies have shown that NETs are abundantly produced in periodontitis[24,25]. During the process of NETosis, DNA-bound histones are citrullinated by the human peptidylarginine deiminases, leading to a change in charge and de-condensation of the DNA[26]. Of note, histones are known to have different roles in NET formation. On the one hand, the positive charge of histones is needed for their bacteriocidal effects. On the other hand, Li and colleagues have shown that citrullination of histone H3 by the human peptidylarginine deiminase 4 (PAD4) is essential for bacterial killing in NETs[27]. The process of NETosis can be artificially induced by addition of Phorbol 12-Myristate 13-Acetate (PMA), as shown in Figure 4a, b (see also Figure S2). We exposed PPAD-proficient and PPAD-deficient *P. gingivalis* to neutrophils undergoing NETosis and observed higher NETosis in both infection situations compared to the uninfected PMA-activated neutrophils. However, a greater number of intact neutrophil nuclei was noticed for PPAD-proficient bacteria compared to the PPAD-deficient bacteria (Fig. 4c, d). This indicates that PPAD activity can impair the bacteria-induced NETosis. Consistent with this view, higher numbers of PPAD-deficient bacteria were observed to be trapped in NETs (Fig. 4c, d) and eliminated upon capture (Fig. 4e). The exact mechanisms by which PPAD could interfere with NET formation are currently unknown and should be a topic of future investigations. A possible explanation could be that the higher levels of secreted protease activity produced by the PPAD-proficient bacteria have a negative impact on the NET formation, for example by degrading certain human proteins needed for DNA decondensation.

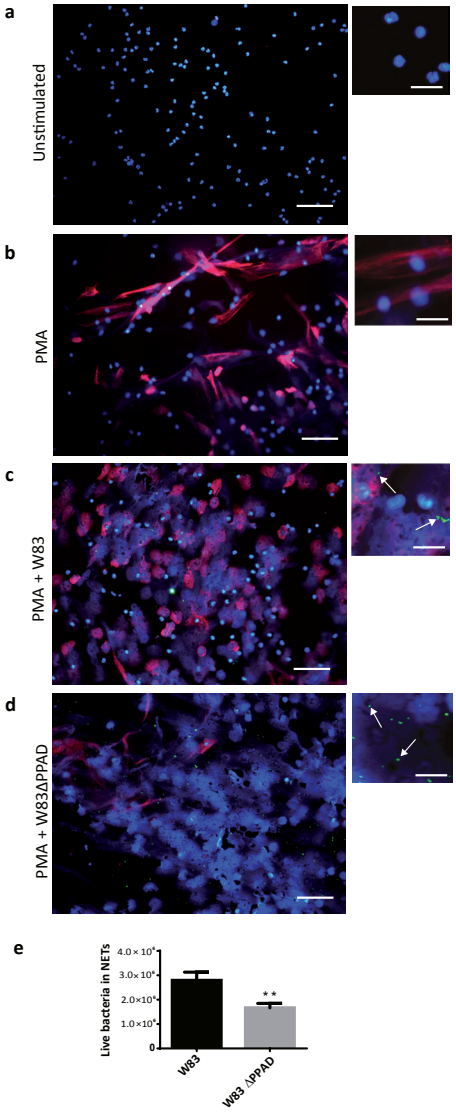


Figure 4: PPAD impacts on histone H3 citrullination and allows *P. gingivalis* to evade and survive capture in neutrophil extracellular traps (NETs). a-d, Representative fluorescence microscopy images of NETosis and citrullinated histone H3 levels in the presence of *P. gingivalis*. PMA was applied at a concentration of 20 nM to induce NETosis. DNA was stained with DAPI (blue), *P. gingivalis* was labeled with FITC (green), and citrullinated histone H3 (citH3; red) was visualized with a specific antibody **(a-d**; scale bars, 200 µm and 50 µm in enlarged images). **e,** Quantification of live bacteria present in isolated NETs.

Histones are critical actors in capture and killing of bacteria in the NETs, and especially the arginine-rich histones directly disrupt the bacterial cell membrane by virtue of their positive charge[21]. We therefore inspected histone H3 citrullination in neutrophils undergoing NETosis, which revealed a strong PPAD-dependent citrullination of this antibacterial agent (Fig. 4c, d). This result was subsequently validated by incubating purified histone H3 with the recombinant PPAD, which led to histone H3 citrullination as shown by Western blotting and mass spectrometry (Fig. 5a, b, Fig. S3a, b). Compared to the purified human peptidylarginine deiminase 2 (PAD2), PPAD showed a somewhat lower citrullinating activity on purified histone H3 that correlated with the citrullination of only one arginine residue (Arg73), whereas human PAD2 was capable of citrullinating up to nine different arginine residues in histone H3 (Fig. 5a).

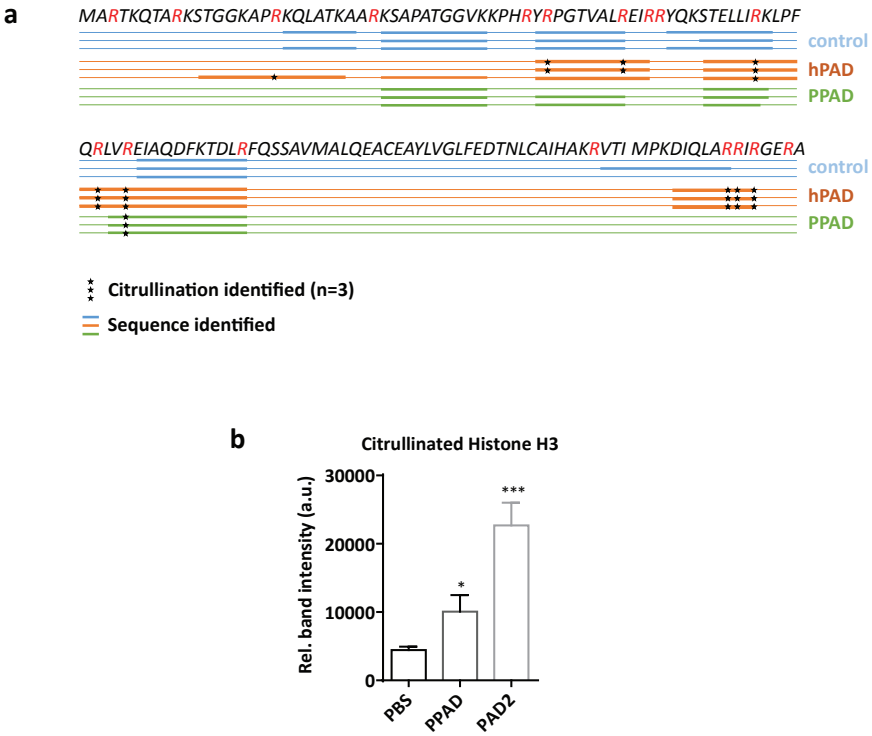


Figure 5: PPAD citrullinates histone H3. *In vitro* citrullination of histone H3. Citrullination by human PAD2 was used as a positive control. **a**, Schematic representation of citrullinated arginine residues in histone H3 upon incubation with PPAD or PAD2, as determined by mass spectrometry. **b**, Western blot analysis of citrullinated histone H3. Quantification of band intensity in three independent experiments is shown. **P*<0.05, ****P*<0.001, two-tailed unpaired Student's *t*-tests. Data are presented as mean values ± SD.

Even so, in terms of citrullination of the NETs-associated histone H3, the impact of PPAD was much higher than that of any other human PAD released by neutrophils undergoing NETosis (Fig. 4b-d). These findings are fully consistent with the previously published observation that citrullinated histone H3 is abundantly detectable in inflamed periodontal tissue[28]. Thus, *P. gingivalis* is capable of neutralizing a major NETs-associated histone implicated in bacterial elimination in the periodontium, where PPAD is clearly detectable (Fig.1b).

PPAD citrullinates human lysozyme-derived peptide LP9, neutralizing its antibacterial activity

The bacterial cell wall-degrading enzyme lysozyme is an important contributor to human innate immunity. This enzyme, abundantly present in our saliva, is also produced by neutrophils[29,30]. It acts in two different modes: the full-size protein has a muramidase activity that degrades peptidoglycan, leading to bacterial lysis. In addition, degradation products of lysozyme act as cationic antimicrobial peptides (CAMPs), as was shown for the LP9 peptide ($_{107}$R-A-W-V-A-W-R-N-R$_{115}$)[31]. LP9 introduces pores into the bacterial cell membrane by electrostatic interaction, leading to bacterial death. Presumably this relates to LP9's three arginine residues. We therefore tested whether PPAD can neutralize LP9 by citrullination, thereby abrogating its bactericidal activity towards LP9-susceptible bacteria. This is indeed the case as mass spectrometry showed that PPAD can convert all three arginines of LP9 to citrulline (Fig. 6a). Concomitantly, citrullination reduced LP9's bactericidal activity as demonstrated with the LP9-susceptible indicator *Bacillus subtilis* (Fig. 6b). This shows that PPAD can even neutralize CAMPs, which belong to our most effective defenses against bacterial pathogens. Notably, PPAD-proficient and PPAD-deficient *P. gingivalis* strains are not susceptible to LP9 (Fig. S4). This shows that PPAD is not the only factor that protects *P. gingivalis* against LP9 activity. In fact, this finding is in agreement with the previous observation that gingipains play an important role in the de-activation of CAMPs by proteolytic degradation[32,33].

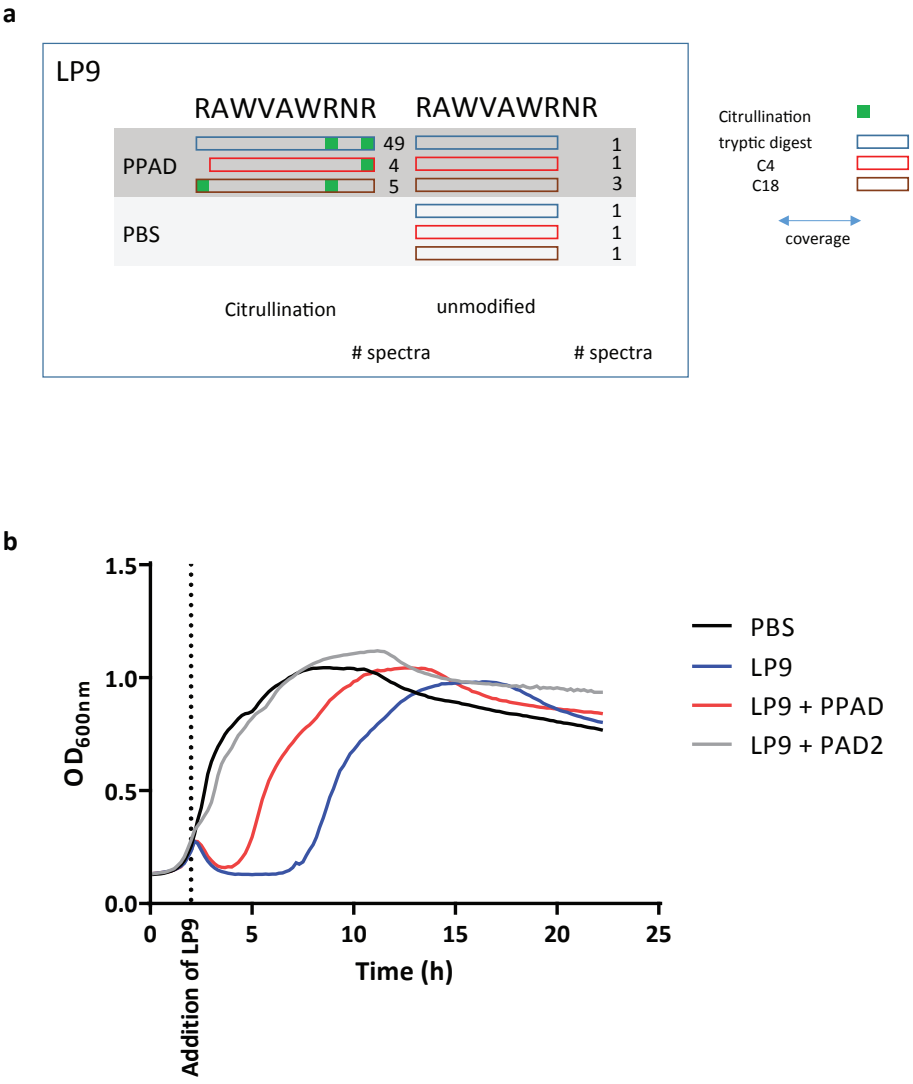


Figure 6: PPAD citrullinates human lysozyme-derived peptide LP9, neutralizing its antibacterial activity. a, Arginine residues in the LP9 peptide (R-A-W-V-A-W-R-N-R) are citrullinated by PPAD, as determined by mass spectrometry. Blue, red and brown rectangles mark the outcomes from three distinct analytical approaches: tryptic digest, C4 exclusion filtration and C18 inclusion filtration, respectively. **b**, Citrullination of LP9 by PPAD or PAD2 impairs the antibacterial activity of LP9. Citrullinated LP9 exhibits significantly reduced growth inhibition of the indicator bacterium *B. subtilis*. Results in (**b**) are representative of three independent experiments with three technical replicates per experiment.

PPAD is a critical virulence factor of *P. gingivalis*

While the above studies show that PPAD targets innate immunity at three different levels, an important question that remained to be addressed was whether it contributes *in vivo* to the virulence of *P. gingivalis*. This was investigated using larvae of the wax moth *Galleria mellonella*, because this infection model only possesses an innate immune system. Hemocytes, the main innate immune cells of *G. mellonella*, closely resemble human neutrophils since they employ the same defense mechanisms, in particular phagocytosis and NETosis[34]. As shown in Figure 7, *G. mellonella* larvae are less susceptible to injected PPAD-deficient *P. gingivalis* than to the wild-type bacteria, whereas heat-killed *P. gingivalis* bacteria do not affect larval viability. This observation is fully in line with the here proposed role of PPAD as an immune evasion factor.

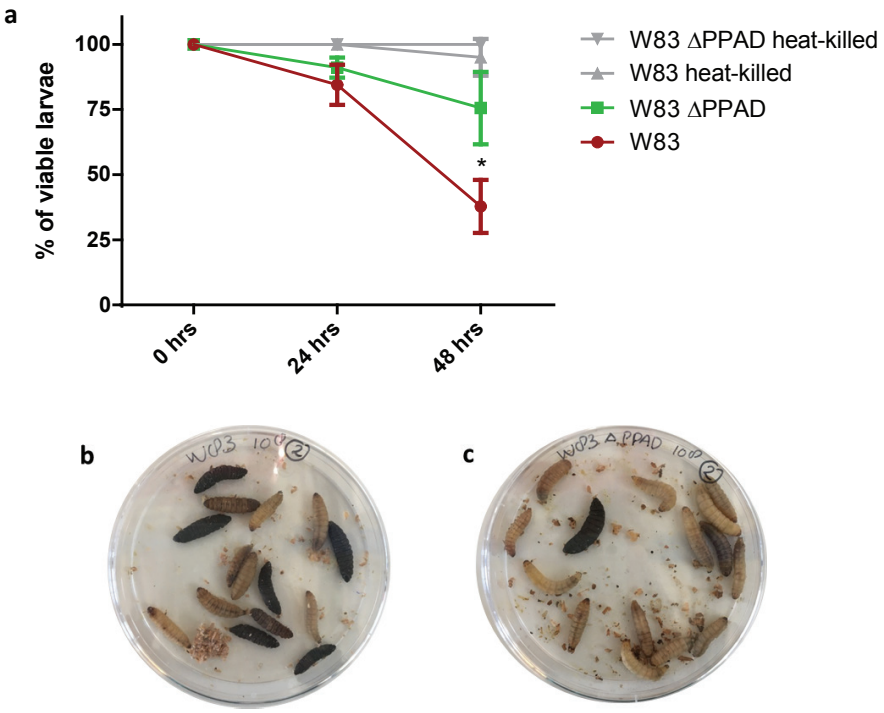


Figure 7: PPAD is a critical virulence factor of *P. gingivalis*. Viability of *Galleria mellonella* larvae was measured 24 h and 48 h after infection with *P. gingivalis*. **a**, The larvae were significantly less susceptible to *P. gingivalis* W83 ΔPPAD than to the wild-type strain W83. Heat-killed bacteria were used as a negative control. Data in (**a**) are means of three biological replicates (n=15). **b-c**, Representative images of *G. mellonella* larvae infected with *P. gingivalis* W83 (**b)** or W83 ΔPPAD (**c**) **P*<0.05, two-tailed unpaired Student's *t*-tests.

Conclusion

Altogether, our present findings show for the first time that the virulence factor PPAD of the oral pathogen *P. gingivalis* defuses antibacterial neutrophil insults at three distinct levels, namely phagocytosis, NETosis and CAMP activity. This identifies PPAD as a major agent in the evasion of human innate immunity, a view that is supported by studies from Potempa and co-workers showing PPAD-dependent citrullination of the complement system[35]. Importantly, an essential role of PPAD in immune evasion explains why this enzyme is invariantly produced by all of the over 100 clinical isolates of *P. gingivalis* investigated to date[8,36].

Materials and Methods

Immunohistochemistry. Immunohistochemical staining of PPAD was performed as described before[28]. Briefly, human paraffin-embedded gingival tissues were collected from *P. gingivalis*-colonized periodontitis patients at the dentistry department of the University Medical Center Groningen. Deparaffinisation of 5 µm sections was performed by several xylene, ethanol and water washes. Endogenous peroxidase activity was inhibited by addition of hydrogen peroxide in methanol, followed by blocking of non-specific antibody binding with 1% bovine serum albumin and 1% normal goat serum in PBS. Next, samples were stained, either with an in-house produced PPAD-specific antibody[7,8] or with the respective pre-immune serum (1:100 in PBS, 1 h). Upon removal of excessive primary antibody by PBS, a secondary goat-anti-rabbit IgG-HRP antibody (P0448; Dako, Santa Clara, USA) was added at a concentration of 1:100 in PBS for 45 min, followed by washing and a developing reaction using a DAB kit (K3467; Dako). Sections were counter-stained with haematoxylin and mounted with glycerine before microscopic evaluation.

***P. gingivalis* culture.** The *P. gingivalis* reference strain W83, as well as the respective PPAD-deficient mutant (W83ΔPPAD)[37] were grown as described before[6]. For infection experiments, liquid cultures were grown until stationary phase, which was reached after ~24 h. For several experiments, inoculation was performed by diluting bacterial glycerol stocks stored at -80°C in a 1:100 ratio into fresh brain heart infusion (BHI) medium (Oxoid, Basingstoke, UK).

Neutrophil isolation. Neutrophils were freshly isolated from four healthy donors (two females aged 27 and 34; two males aged 28 and 39), who had been medically examined. A lymphoprep™ (Stem cell technologies, Vancouver, Canada) buffer was used to separate cell types. EDTA-blood was first diluted 1:1 with PBS and then put gently on top of a volume of lymphoprep (blood – lymphoprep ratio 2:1). Samples were centrifuged at 2500 rpm at room temperature (RT) for 20 min without brake to not disrupt the separated cell layers. The plasma, lymphoprep and peripheral blood mononuclear cells were removed, and a layer of erythrocytes and neutrophils remained. The erythrocytes in this mixture

were lysed by adding ammonium chloride 0.8 %, 1 mM EDTA, pH 7.4 and shaking for 10 min on ice. After another 2500 rpm centrifugation for 3 min, the lysed erythrocytes were removed. These two steps were repeated once more to obtain a pellet of purified neutrophils.

OMV and PPAD preparation. *P. gingivalis* cultures in late exponential phase were used for OMV collection. A first centrifugation step at 8000 x g and 4°C for 20 min was performed to separate cells from OMV-containing supernatant. The latter was subjected to ultracentrifugation at 100,000 x g and 4°C, for 3 h in an Optima MAX-XP ultracentrifuge 261 (Beckman Coulter, Brea, USA) using an MLA-80 fixed angle rotor. The pellet containing the OMVs was resuspended in PBS, and aliquots were frozen at -80°C before use. Protein quantification was performed using a bicinchoninic acid (BCA) protein assay (Pierce, Waltham, USA) according to the manufacturer's instructions, with the addition of 2.0% SDS to solubilise proteins. An amount of 16 µg of protein was used for the phagocytosis rescue experiment. Protein precipitation with 10% TCA was performed as described before[6] to concentrate vesicles for Western blot analysis. Recombinant PPAD was purified from *Lactococcus lactis* as previously described[7,8].

Neutrophil infections. For neutrophil infection experiments followed by Western-blotting or mass spectrometry analyses, 3 x 10^6 neutrophils in 2.5 mL of Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Waltham, USA) with 2 mM L-glutamine and 10% autologous donor serum were seeded in each well of a 6-well plate. Phagocytosis experiments were carried out with 5 x 10^5 neutrophils in 500 µL of medium in 24-well plates. The neutrophils were allowed to rest on the plate at 37°C and 5% CO_2 for 1 h. When required, 100 µL supernatant of W83, 100 µL BHI medium, 2.5 µg recombinant PPAD, 16 µg OMVs of W83 or 100 µL PBS were added to the neutrophil suspension and incubation was continued for 30 min. Subsequently, *P. gingivalis* was added at a multiplicity of infection (MOI) of 100. The neutrophils were exposed to the bacteria for 90 min. Extracellular bacteria were then removed and the neutrophil layer was washed once with PBS before addition of NP40 lysis buffer (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris pH 8.0) with cOmplete™ mini protease inhibitor (Roche, Basel, Switzerland).

Phagocytosis assay. To determine whether PPAD impacts on the association and/or internalization of *P. gingivalis* in neutrophils as defined by Lei et al.[38], a flow-cytometry based method was used as described previously[39]. Briefly, a liquid bacterial culture was centrifuged for 10 min at 7000 x g at 4°C and washed once in PBS before resuspending the bacterial pellet in 0.5 M $NaHCO_3$, pH 8.0 to a concentration of 2.5 x 10^9 colony-forming units/mL before addition of fluorescein (FITC; Invitrogen, Carlsbad, USA). Bacterial concentrations were approximated by optical density readings at 600 nm according to a standard curve for each strain used.

A FITC concentration of 0.15 mg/mL was used for staining *P. gingivalis* W83 and W83 ΔPPAD[39,40]. The tubes with bacteria and FITC were subsequently incubated in the dark for 30 min at RT in a tube rotator. The bacteria were pelleted at 7000 x g for 5 min and the pellet was washed 3 times with PBS to remove unbound FITC. Finally, the bacteria were resuspended to the desired concentration in RPMI 1640/FBS 10%/2 mM L-glutamine.

To measure the bacterial internalization rate, the extracellular fluorescence (representing associated but not internalized bacteria) was quenched using 0.2% trypan blue (Thermo Fisher Scientific, Waltham, USA). Subsequently, two washing steps with PBS were performed to remove excessive trypan blue. Both quenched and non-quenched cell samples were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, USA) for 15 min prior to flow cytometric analyses and visualization by fluorescence microscopy.

An Accuri™ C6 Flow cytometer was used to measure the mean fluorescence intensity (MFI) of the FITC-positive cells. The gating strategy to include only neutrophils in our analysis is shown in Figure S1e-g. FITC-positive cells were identified by setting a fluorescence threshold in an uninfected neutrophil control sample, next to the auto-fluorescence peak, as shown in Figure S1h-j. The association index of each *P. gingivalis* strain was calculated by multiplying the percentage of FITC-positive cells with associated bacteria (i.e. intracellular + extracellularly bound bacteria) with the MFI of these cells, divided by 100, as previously described[41]. The internalization index of each *P. gingivalis* strain was calculated by multiplying the percentage of cells with internalized bacteria (cells positive for FITC after trypan blue quenching) with the MFI of these cells, divided by 100 [38]. For microscopic analyses, 10 µL of the fixed cells was mounted on microscopy slides and visualised with an Axio Observer.Z1 fluorescence microscope (Zeiss, Jena, Germany) using a 40x or 65x magnification. Images were recorded using an Axio Cam MRm Rev. 3 camera with FireWire.

LDS-PAGE. Lithium dodecyl sulphate (LDS)-PAGE was performed using 10% NuPAGE gels (Invitrogen, Carlsbad, USA). Protein concentrations of cell lysates were determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) and frozen at -20 °C until further use. Equal amounts of protein samples were incubated with LDS sample buffer for 10 min at 95°C, separated by LDS-PAGE, and either stained with SimplyBlue™ SafeStain (Life Technologies, Carlsbad, USA) or processed further for Western blotting.

Western blotting. For Western blotting, proteins were transferred from the gel to a nitrocellulose membrane (Whatman, Buckinghamshire, UK) by semi-dry blotting. The transfer was performed at 200 mA for 75 min in the presence of methanol-containing buffers. Upon transfer, the non-specific binding was blocked overnight at 4°C with 5% skim milk (Oxoid, Basingstoke, UK) in PBS. Afterwards the blot was rinsed once with PBS-T to remove residual skim milk. Primary rabbit-anti-RgpA/B, rabbit-anti-PPAD antibodies[7,8] or anti-histone H3 (Abcam, ab18521) in PBS-T (1:2000) were added, and the blot was incubated for 1 h at RT. After removing the non-bound primary antibodies by 4 washes with PBS-T, the blot was incubated with IRDye 800CW goat-anti-rabbit antibody (LI-COR Biosciences, Lincoln, USA) in PBS-T (1:10,000) protected from light for 45 min. Lastly, background was reduced by washing 4 times with PBS-T and subsequently washing twice with PBS to remove the tween. Fluorescence was measured with the LI-COR ODYSSEY® (LI-COR Biosciences, Lincoln, USA) infrared imaging system and subsequently quantified with ImageJ (National Institutes of Health, Bethesda, USA).

Protease activity assay. *P. gingivalis* was grown in BHI medium until stationary phase, and the growth medium was separated from the cells by centrifugation at 7000 x g for 10 min. Recombinant human histone H3 (0.5 µg; New England Biolabs, Ipswich, MA, USA) was incubated with 7.5 µL of the growth medium fraction for 1, 5, 10, 15, 20 and 30 min at 37°C. Fresh BHI medium (7.5 µL) was used as a negative control. The resulting protein samples were analyzed by Western blotting, as described above.

Mass spectrometry of neutrophils. Neutrophil lysates were processed for mass spectrometry analysis as described before[42]. Briefly, proteins were bound to Strataclean resins (Agilent Technologies, Santa Clara, USA) and subsequently alkylated, reduced and digested by trypsin. Resulting peptides were purified by C18 stage-tip purification (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol and dried until further use.

Purified peptides were analyzed by reversed phase liquid chromatography (LC) electrospray ionization (ESI) MS/MS using an Orbitrap Elite (Thermo Fisher Scientific, Waltham, USA). In brief, in-house self-packed nano-LC columns (20 cm) were used to perform LC with an EASY-nLC 1200 system (Thermo Fisher Scientific, Waltham, USA). The peptides were loaded with buffer A (0.1% acetic acid (v/v)) and subsequently eluted in 156 min using a 1% to 99% non-linear gradient with buffer B (0.1% acetic acid (v/v), 94.9% acetonitrile). After injection into the MS, a full scan was recorded in the Orbitrap with a resolution of 60,000. The twenty most abundant precursor ions were consecutively isolated in the linear ion trap and fragmented via collision-induced dissociation (CID). Unassigned charge states as well as singly charged ions were rejected and the lock mass option was enabled.

Database searching was done with Sorcerer-SEQUEST 4 (Sage-N Research, Milpitas, USA). After extraction from the raw files, *.dta files were searched with Sequest against a target-decoy database with a set of common laboratory contaminants. A database for the respective peptide/protein search was created from the published genome sequences of the W83 strain and the human genome which were downloaded from Uniprot (http://www.uniprot.org) on 14/07/2016. The created database contained a total number of 148,472 proteins. Database search was based on a strict trypsin digestion with two missed cleavages permitted. No fixed modifications were considered. Oxidation of methionine, carbamidomethylation of cysteine and citrullination of arginine were considered as variable modifications. The mass tolerance for precursor ions was set to 10 ppm and the mass tolerance for fragment ions to 1 Da. Validation of MS/MS based peptide and protein identification was performed with Scaffold v.4 (Proteome Software, Portland, USA). A false discovery rate (FDR) of 0.1% was set for filtering the data. Protein identifications were accepted if at least 2 identified peptides were detected with the above-mentioned filter criteria in 2 out of 3 biological replicates. Protein data were exported from Scaffold and further curated in Microsoft Excel 2013 before further analysis. The neutrophil mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (https://www.ebi.ac.uk/pride/) with the dataset identifier PXD010798.

Quantitative values of protein abundances in neutrophil samples were obtained by summing up all spectra associated with a specific protein within a sample, which includes also those spectra that

are shared with other proteins. To allow comparisons, spectral counts were normalized by applying a scaling factor for each sample to each protein adjusting the values to normalized quantitative values.

Mass spectrometry of histone H3 and LP9. Recombinant human histone H3 (0.5 µg; New England Biolabs, Ipswich, MA, USA) was incubated with recombinant PPAD (0.25 µg) overnight at 37°C. Proteins were separated by LDS-PAGE and stained with SimplyBlue™ SafeStain as described above. Histone H3-corresponding bands (Fig. S3b) were excised from the gel, dried and further processed by trypsin digestion as described above.

LP9 was synthesized at EMC microcollections GmbH (Tübingen, Germany). The LP9 peptide (0.5 µg) was incubated with recombinant PPAD (0.25 µg) overnight at 37°C. Subsequently, the samples were processed by three different methods: (1) Trypsin digestion. Samples were alkylated, reduced, digested by trypsin and purified by C18 ZipTip purification as described above. (2) C4 Exclusion of PPAD. PPAD was excluded by C4 ZipTip filtration using a slight modification of the manufacturer's protocol. Upon binding of PPAD to the ZipTip, the PPAD-containing tip was discarded and the LP9-containing flow-through was further processed by C18 ZipTip filtration. (3) C18 inclusion of LP9. The LP9 peptides were immediately purified by C18 ZipTip filtration following the manufacturer's protocol.

Purified peptides were analyzed by reversed phase LC-ESI-MS/MS using an Orbitrap Elite (Thermo Fisher Scientific, Waltham, USA). In brief, in-house self-packed nano-LC columns (20 cm; packed with Aeris Peptide material 3.6µm XB- C18-100Å) were used to perform LC with an EASY-nLC 1200 system (Thermo Fisher Scientific, Waltham, USA). The peptides were loaded with buffer A (0.1% acetic acid (v/v)) and subsequently eluted in 80 min using a non-linear gradient of 1% to 99% with buffer B (0.1% acetic acid (v/v), 94.9% acetonitrile). After injection into the MS, a full scan was recorded in the Orbitrap with a resolution of 60,000. The twenty most abundant precursor ions were consecutively isolated in the linear ion trap and fragmented via collision-induced dissociation (CID). Unassigned charge states as well as singly charged ions were rejected and the lock mass option was enabled.

Database searching for the histone H3 and LP9 analyses was done with Sorcerer-SEQUEST 4 (Sage-N Research, Milpitas, USA). After extraction from the raw files, *.dta files were searched with Sequest against a target-decoy database with a set of common laboratory contaminants. For the peptide/protein search the sequence of LP9 was added to the database that was used for analysis of the neutrophil MS data, and the database search was performed based on the same criteria as described above. For the histone H3 analysis, SEQUEST identifications required XCorr scores of greater than 2.2, 3.3 and 3.8 for doubly, triply and all higher charged peptides, respectively. For the LP9 analysis, SEQUEST identifications required XCorr scores of greater than 2.7, 3.5 and 3.5 for doubly, triply and all higher charged peptides. Protein data were exported from Scaffold. The histone H3 and LP9 mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (https://www.ebi.ac.uk/pride/) with the dataset identifier PXD009081. Spectra and fragmentation tables of the peptides identified to be citrullinated are presented in Supplemental Figure S5.

Immunofluorescence microscopy of NET formation. For microscopic analysis of infected neutrophils, sterile 12 mm diameter coverslips (Menzel-Gläser, Braunschweig, Germany) were placed into 24-well plates (Corning, Corning, USA). 2.5 x 10^5 neutrophils in 500 µL RPMI 1640 medium were added to each well. To let the neutrophils adhere to the coverslips, plates were incubated for 1 h at 37°C and 5% CO_2. Subsequently, cells were stimulated for 1 h with 20 mM Phorbol 12-Myristate 13-Acetate (PMA, Sigma-Aldrich, St. Louis, USA) to induce NETosis and then infected with *P. gingivalis* at an MOI of 100 for 90 min. Upon infection, 500 µl of 8% PFA was added to each well to reach a final concentration of 4% PFA to fix the cells. Plates were stored at 4°C in the dark, and immunofluorescence staining was performed on the following day. For this, the fixative solution was removed, and the cell layer was washed carefully one time with PBS. A blocking step was performed incubating cells at RT with 2% BSA in PBS for 1 h. Citrullinated histone H3 in NETs was stained with a rabbit-anti-citrullinated histone H3 antibody (Abcam, ab5103, 1:250) and incubated for 1 h at RT in PBS, 0.05% Tween-20, 0.5% BSA. Coverslips were washed with PBS before adding secondary antibodies. The Alexa Fluor 568 goat-anti-rabbit antibody (Invitrogen, A11011, 1:400) was used to visualize the primary antibodies. Secondary antibodies were added in PBS with DAPI (Roche, 10236276001, 1:5000) and incubated for 30 min before mounting the coverslips in citifluor (Citifluor, Hatfield, USA). Slides were then analyzed using a Leica DFC450 C fluorescence microscope with the Leica Application Suite software version 4.2.0.

NET survival assay. NETosis was induced, and *P. gingivalis* was added to the NETs as described above, with the modification that no cover slips were placed into the wells. Upon 90 min of infection, NETs were isolated as described previously[43]. Subsequently, different dilutions of bacteria trapped in the NETs were plated on Blood Agar Base No. 2 (BA2) plates (Oxoid, Basingstoke, UK). The plates were incubated for 5 days at 37°C under anaerobic conditions, and *P. gingivalis* colonies were counted.

Citrullination of LP9 and killing assay. *Bacillus subtilis* strain 168 was grown overnight in BHI broth (Oxoid, Basingstoke, UK) shaking at 37°C. The culture was diluted to an optical density at 600 nm of 0.1, and 100 µl of this suspension was pipetted in each well of a 96-well plate. Bacteria were grown for 2 h shaking at 37°C in a Biotek Synergy 2 Microplate Reader (Biotek Instruments Inc., Winooski, USA) until they reached exponential phase, and LP9 (in PBS) was added at a final concentration of 200 µg/mL. To investigate the effect of citrullination on its activity, LP9 was pre-incubated with PPAD or hPAD2 overnight at 37°C before addition. Bacterial growth was monitored until stationary phase and the respective growth curves were plotted with GraphPad Prism version 6 (GraphPad Software, La Jolla, USA). The effect of LP9 on exponentially growing cells was determined by measuring the growth delay of *B. subtilis* upon addition of LP9. The same procedure was applied for the killing assay of *P. gingivalis*. However, for *P. gingivalis*, standing cultures were grown for 48 h at 37°C.

***In vivo Galleria mellonella* survival assay.** Larvae of *G. mellonella* were injected with the *P. gingivalis* W83 strain or the respective PPAD-deficient mutant. Bacteria were injected into the last proleg at a volume of 10 µL using a HumaPen Luxura HD (Eli Lilly, Indianapolis, USA). Viability was scored by one

trained person at 24 h and 48 h post-infection based on pigmentation and mobility. To assess virulence of the investigated *P. gingivalis* strains, the larvae were infected with an amount of 10^8 PBS-washed bacteria. Heat-killed bacteria (30 min, 90°C) were used as a negative control.

Statistical analyses. Statistical analyses were performed with GraphPad Prism version 6 (GraphPad Software, La Jolla, USA) or with Scaffold v.4 (Proteome Software, Portland, USA). Two groups were compared by performing an unpaired, two-tailed Student's t-test. The Fisher's exact test was used to assess significance of differences in normalized spectral counts of neutrophil proteins detected by MS. Significance was defined as a p-value lower than or equal to 0.05.

Medical Ethical Committee Approval. Blood donations from healthy volunteers were collected with approval of the medical ethics committee of the University Medical Center Groningen (UMCG; approval no. Metc2012-375). All blood donations were obtained after written informed consent from all volunteers, and adhering to the Helsinki Guidelines.

Biological and chemical safety. *P. gingivalis* was handled following appropriate safety and containment procedures for biosafety level 2 microbiological agents. All experiments involving human cells were performed under appropriate safety conditions. All chemicals and reagents applied in this study were handled according to local guidelines for safe usage and protection of the environment.

Data availability. The mass spectrometry data are deposited in the ProteomeXchange repository PRIDE (https://www.ebi.ac.uk/pride/) with the dataset identifiers PXD010798 (Username for review: reviewer86614@ebi.ac.uk; Password: gqdbDb44) and PXD009081 (Username for review: reviewer43245@ebi.ac.uk; Password: vSEoj2i1).

List of Supplemental Material

Table S1 | Overview of phagocytosis-related proteins identified in infected neutrophil samples.
Figure S1 | Supporting data for neutrophil invasion assay.
Figure S2 | Fluorescence microscopy images of NETosis in the presence of *P. gingivalis*
Figure S3 | Histone H3 citrullination by PPAD.
Figure S4 |Susceptibility of *P. gingivalis* to LP9.
Figure S5 | Spectra and fragmentation tables of the peptides identified to be citrullinated.

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References

1. Tonetti, M. S., Jepsen, S., Jin, L. & Otomo-Corgel, J. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. *J. Clin. Periodontol.* 44, 456–462 (2017).
2. Hajishengallis, G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* 15, 30–44 (2014).
3. Cortés-Vieyra, R., Rosales, C. & Uribe-Querol, E. Neutrophil Functions in Periodontal Homeostasis. *J. Immunol. Res.* 2016, 1396106–9 (2016).
4. McGraw, W. T., Potempa, J., Farley, D. & Travis, J. Purification, characterization, and sequence analysis of a potential virulence factor from *Porphyromonas gingivalis*, peptidylarginine deiminase. *Infect. Immun.* 67, 3248–3256 (1999).
5. Wegner, N. *et al.* Peptidylarginine deiminase from *Porphyromonas gingivalis* citrullinates human fibrinogen and α-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum.* 62, 2662–72 (2010).
6. Stobernack, T. *et al.* Extracellular Proteome and Citrullinome of the Oral Pathogen *Porphyromonas gingivalis*. *J. Proteome Res*. 15, 4532–4543 (2016).
7. Gabarrini, G. *et al.* Conserved Citrullinating Exoenzymes in Porphyromonas Species. *J. Dent. Res* 97, 556–562 (2018).
8. Gabarrini, G. *et al.* There's no place like OM: Vesicular sorting and secretion of the peptidylarginine deiminase of *Porphyromonas gingivalis*. *Virulence* 9, 456–464 (2018).
9. de Smit, M. *et al.* Periodontitis in established rheumatoid arthritis patients: a cross-sectional clinical, microbiological and serological study. *Arthritis Res. Ther.* 14, R222 (2012).
10. Berthelot, J.-M. & Le Goff, B. Rheumatoid arthritis and periodontal disease. *Jt., Bone, Spine* 77, 537–41 (2010).
11. Lundberg, K., Wegner, N., Yucel-Lindberg, T. & Venables, P. J. Periodontitis in RA—the citrullinated enolase connection. *Nat. Rev. Rheumatol.* 6, 727–730 (2010).
12. Neiders, M. E. *et al.* Heterogeneity of virulence among strains of *Bacteroides gingivalis*. 24, 192–198 (1989).
13. Potempa, J., Sroka, A., Imamura, T. & Travis, J. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: structure, function and assembly of multidomain protein complexes. *Curr. Protein Pept. Sci.* 4, 397–407 (2003).
14. Curtis, M. A. *et al.* Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. *Infect. Immun.* 67, 3816–3823 (1999).
15. Otsuka, A. *et al.* Dynamin 2 is required for actin assembly in phagocytosis in Sertoli cells. *Biochem. Biophys. Res. Commun*. 378, 478–482 (2009).
16. May, R. C., Caron, E., Hall, A. & Machesky, L. M. Involvement of the Arp2/3 complex in phagocytosis mediated by FcgammaR or CR3. *Nat. Cell Biol.* 2, 246–248 (2000).
17. Park, H. & Cox, D. Cdc42 regulates Fc gamma receptor-mediated phagocytosis through the activation and phosphorylation of Wiskott-Aldrich syndrome protein (WASP) and neural-WASP. *Mol. Biol. Cell* 20, 4500–4508 (2009).
18. Löfgren, R., Ng-Sikorski, J., Sjölander, A. & Andersson, T. Beta 2 integrin engagement triggers actin polymerization and phosphatidylinositol trisphosphate formation in non-adherent human neutrophils. *J. Cell Biol.* 123**,** 1597–1605 (1993).
19. Mazzone, A. & Ricevuti, G. Leukocyte CD11/CD18 integrins: biological and clinical relevance. *Haematologica* 80, 161–175 (1995).
20. Ambruso, D. R., Cusack, N. & Thurman, G. NADPH oxidase activity of neutrophil specific granules: requirements for cytosolic components and evidence of assembly during cell activation. *Mol. Genet. Metab.* 81, 313–321 (2004).
21. Hoeksema, M., van Eijk, M., Haagsman, H. P. & Hartshorn, K. L. Histones as mediators of host defense, inflammation and thrombosis. *Future Microbiol.* 11, 441–453 (2016).
22. Papayannopoulos, V. Neutrophil extracellular traps in immunity and disease. *Nat. Rev. Immunol.* 18, 134–147 (2018).
23. Sørensen, O. E. & Borregaard, N. Neutrophil extracellular traps - the dark side of neutrophils. *J. Clin. Invest.* 126, 1612–1620 (2016).

24. White, P. C., Chicca, I. J., Cooper, P. R., Milward, M. R. & Chapple, I. L. C. Neutrophil Extracellular Traps in Periodontitis: A Web of Intrigue. *J. Dent. Res* 95, 26–34 (2015).

25. Vitkov, L., Hartl, D., Minnich, B. & Hannig, M. Janus-Faced Neutrophil Extracellular Traps in Periodontitis. *Front. Immunol.* 8, 1404 (2017).

26. Wang, Y. *et al.* Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J. Cell Biol.* 184, 205–213 (2009).

27. Li, P. *et al.* PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J. Exp. Med.* 207**,** 1853–1862 (2010).

28. Janssen, K. M. J. *et al.* Autoantibodies against citrullinated histone H3 in rheumatoid arthritis and periodontitis patients. *J. Clin. Periodontol.* 44**,** 577–584 (2017).

29. Lollike, K., Kjeldsen, L., Sengeløv, H. & Borregaard, N. Lysozyme in human neutrophils and plasma. A parameter of myelopoietic activity. *Leukemia* 9, 159–164 (1995).

30. Fábián, T. K., Hermann, P., Beck, A., Fejérdy, P. & Fábián, G. Salivary defense proteins: their network and role in innate and acquired oral immunity. *Int. J. Mol. Sci.* 13, 4295–4320 (2012).

31. Herbert, S. *et al.* Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. *PLoS Pathog.* 3, e102 (2007).

32. Gutner, M., Chaushu, S., Balter, D. & Bachrach, G. Saliva enables the antimicrobial activity of LL-37 in the presence of proteases of *Porphyromonas gingivalis*. *Infect. Immun.* 77, 5558–5563 (2009).

33. Maisetta, G., Brancatisano, F. L., Esin, S., Campa, M. & Batoni, G. Gingipains produced by *Porphyromonas gingivalis* ATCC49417 degrade human-β-defensin 3 and affect peptide's antibacterial activity in vitro. *Peptides* 32**,** 1073–1077 (2011).

34. Browne, N., Heelan, M. & Kavanagh, K. An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. *Virulence* 4, 597–603 (2013).

35. Bielecka, E. *et al.* Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity. *J. Biol. Chem*. 289**,** 32481–32487 (2014).

36. Gabarrini, G. *et al.* The peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis*. *Sci. Rep.* 5, 13936 (2015).

37. Wegner, N. *et al.* Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. *Immunol. Rev.* 233, 34–54 (2010).

38. Lei, L., Li, H., Yan, F. & Xiao, Y. Hyperlipidemia impaired innate immune response to periodontal pathogen *porphyromonas gingivalis* in apolipoprotein E knockout mice. *PLoS ONE* 8, e71849 (2013).

39. Pathirana, R. D., O'Brien-Simpson, N. M., Visvanathan, K., Hamilton, J. A. & Reynolds, E. C. Flow cytometric analysis of adherence of *Porphyromonas gingivalis* to oral epithelial cells. *Infect. Immun.* 75, 2484–2492 (2007).

40. Igboin, C. O., Griffen, A. L. & Leys, E. J. *Porphyromonas gingivalis* strain diversity. *J. Clin. Microbiol.* 47, 3073–3081 (2009).

41. Rossi, A. & Lord, J. Adiponectin inhibits neutrophil phagocytosis of Escherichia coli by inhibition of PKB and ERK 1/2 MAPK signalling and Mac-1 activation. *PLoS ONE* 8, e69108 (2013).

42. Bonn, F. *et al.* Picking vanished proteins from the void: how to collect and ship/share extremely dilute proteins in a reproducible and highly efficient manner. *Anal. Chem.* 86, 7421–7 (2014).

43. Barrientos, L. *et al.* An improved strategy to recover large fragments of functional human neutrophil extracellular traps. *Front. Immunol.* 4, 166 (2013).

CHAPTER 6

Porphyromonas gingivalis and its secreted peptidylarginine deiminase modulate the proteome of human neutrophils and macrophages

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Abstract

Periodontitis and rheumatoid arthritis (RA) belong to the most prominent inflammatory diseases in the world. Individuals suffering from periodontitis have a higher chance to develop RA, which might be explained by activities of the periodontal pathogen *Porphyromonas gingivalis*. In particular, the latter produces a unique enzyme called *P. gingivalis* peptidylarginine deiminase (PPAD), which citrullinates bacterial and human proteins. In inflamed gingival tissue, *P. gingivalis* is known to be challenged by innate immune cells, but our understanding of the effects of this bacterium and PPAD on such immune cells is thus far incomplete. Therefore, the aim of this study was to determine effects of *P. gingivalis* and PPAD on the proteome and citrullinome of human neutrophils and macrophages. This was achieved by advanced proteomics, including an unlabeled mass spectrometry approach for inspection of the neutrophil proteome and a SILAC mass spectrometry approach for the macrophage proteome. Our results show that PPAD has a strong impact on the proteome of human neutrophils, causing 'down-regulation' of a plethora of host defense proteins. Several of the down-regulated proteins were found to be citrullinated. Importantly, the magnitude of PPAD-specific effects was inversely correlated with the ability of *P. gingivalis* to evade phagocytosis. Altogether, our present observations place the neutrophil in focus for future research on the roles of *P. gingivalis* in RA and other immune-related diseases.

Key words: *Porphyromonas gingivalis*, peptidylarginine deiminase, PPAD, immune evasion, neutrophil, macrophage

Introduction

Rheumatoid arthritis (RA) and periodontitis are among the most prevalent chronic inflammatory diseases in the world, and there is a significant association between them on a clinical and epidemiological level[1-3]. In the case of periodontitis, inflammation is maintained by an on-going bacterial infection, with the Gram-negative anaerobic bacterium *Porphyromonas gingivalis* being one of the most prominent species involved in the disease[4]. To survive in the oral cavity, *P. gingivalis* produces several virulence factors, including potent proteases (called gingipains), fimbriae and capsule polysaccharides[5-7]. A more recently identified virulence factor is the enzyme *P. gingivalis* peptidylarginine deiminase (PPAD), which catalyzes the post-translational citrullination of proteins. PPAD expression is a unique feature of *P. gingivalis* among human pathogens, and it is strictly conserved within the species[8,9].

Citrullination is a modification where positively charged arginine residues in a protein are converted into neutral citrulline residues. This modification is important for several physiological processes in humans, and it is normally catalyzed by human peptidylarginine deiminase (PAD) enzymes[10]. Of note, human PAD enzymes are different from the bacterial PPAD enzyme with respect to sequence, structure and substrate specificity. PPAD is calcium-independent and prefers terminal arginine residues, while human PADs are calcium-dependent and citrullinate both terminal and internal arginine residues[11]. Intriguingly, PPAD is able to citrullinate both bacterial and human proteins[12,13], and the enzyme has been implicated in immune evasion via citrullination of components of the innate immune system, such as the anaphylatoxin C5a, histones and a lysozyme-derived cationic antimicrobial peptide[14,15]. The citrullination of human proteins is believed to contribute to RA, since patients lose their tolerance to citrullinated proteins and develop anti-citrullinated protein antibodies (ACPAs)[16].

In periodontitis, innate immune cells (i.e. neutrophils and macrophages) are the first cells to confront *P. gingivalis*, since they are recruited in high numbers and constantly penetrate the inflamed tissue [17]. These cells employ several defense mechanisms to protect the human host, including phagocytosis, the production of antimicrobial agents (e.g. peptides and reactive oxygen and nitrogen species), and the ejection of neutrophil extracellular traps (NETs). To better understand the possible role(s) of *P. gingivalis* in the association between periodontitis and RA, the present study was aimed at defining the impact of *P. gingivalis* and PPAD on proteostasis and protein citrullination in human neutrophils and macrophages. To this end, we assessed the changes in human neutrophils and macrophages upon challenges with wild-type *P. gingivalis* or PPAD-deficient derivatives using unlabeled and 'stable isotope labeling with amino acids in cell culture' (SILAC) proteomics approaches.

Materials and Methods

***P. gingivalis* culture.** The *P. gingivalis* reference strains W83 and ATCC 33277, as well as the respective PPAD-deficient mutants W83 dPPAD and ATCC 33277 dPPAD[16] were grown as described before[12]. For infection experiments, Brain-Heart-Infusion (BHI) medium was inoculated with bacterial glycerol stocks stored at -80°C in a 1:100 ratio, and bacteria were grown until stationary phase, which was reached after ~24 h.

Human immune cells. Neutrophils were freshly isolated from healthy donors. For this, a lymphoprep™ (Stem cell technologies, Vancouver, Canada) sedimentation approach was used to separate different cell types. EDTA blood was first diluted 1:1 with phosphate-buffered saline (PBS) and then loaded gently on top of a layer of lymphoprep (blood – lymphoprep ratio 2:1) in 50 mL falcon tubes. Subsequently, the samples were centrifuged at 2500 RPM at room temperature (RT) for 20 min. Centrifugation was stopped without braking to avoid disturbance of the layers. After this step, the plasma, lymphoprep and peripheral blood mononuclear cells were removed leaving behind a layer of red blood cells and neutrophils. The red blood cells in this layer were lysed by adding NH_4Cl (0.8% final concentration) and EDTA (1 mM final concentration; pH7.4), and shaking for 10 min on ice. Remnants of the lysed red blood cells were removed by centrifugation (3 min, 2500 RPM), after which the incubation with NH_4Cl and EDTA and centrifugation were repeated once more. The pellet of purified neutrophils thus obtained was used for further experimentation.

THP-1 monocytes were obtained from liquid nitrogen stocks and passaged at least three times before use for experiments. Cells were cultured in 15 mL Roswell Park Memorial Institute (RPMI) 1640 medium (GE healthcare, Chicago, USA), supplemented with 2 mM L- glutamine and 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany) in T75 flasks. The flasks were incubated at 37°C with 5% CO_2 and cells were split every 3-4 days depending on cell density. For the differentiation of THP-1 monocytes to macrophages, 4 x 10^6 cells were plated on 6-well plates in 2.5 mL RPMI 1640 medium, and 5 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, Germany) was added. Plates were incubated for 48 h at 37°C and 5% CO_2. Morphological changes in differentiated macrophages were monitored by microscopy to ensure proper differentiation.

Experimental Design and Statistical Rationale. For neutrophil infection experiments, 3 x 10^6 cells per 2.5 mL of RPMI 1640 medium (Gibco, Waltham, USA) with 2mM L- glutamine and 10% autologous serum were seeded in each well of a 6-well plate. After an hour of settling on the plate at 37°C and 5% CO_2, *P. gingivalis* cells were added, at a multiplicity of infection (MOI) of 100. Cells were infected for 90 min at 37°C and 5% CO_2, and subsequently lysed with NP40 lysis buffer containing cOmplete™ mini protease inhibitor (Roche, Basel, Switzerland)[15]. Each infection experiment was performed in three biological replicates. The number of replicates was selected to ensure that in every condition there are at least two consistent measurements for protein identification.

For the macrophage infection experiments, a total of 4 x 10^6 macrophages was infected with *P. gingivalis* cells using an MOI of 100 and incubation was continued at 37°C and 5% CO_2 for 22 h [18]. These conditions led to an infection coverage of approximately 60% (Fig. S1b). Medium was removed and the attached macrophages were washed with PBS. The remaining cells and internalized bacteria were lysed with NP40 lysis buffer supplemented with cOmplete™ mini protease inhibitor cocktail (Roche, Basel, Switzerland). Each infection experiment was performed in three biological replicates to ensure that there are at least two consistent measurements for protein identification in every condition.

Phagocytosis assay. To determine whether PPAD impacts on the association and/or internalization of *P. gingivalis* in neutrophils and macrophages, a flow-cytometry based method was used as described previously[19]. Briefly, a bacterial culture in BHI was centrifuged for 10 min at 7000 x g at 4°C and washed once in PBS before resuspending the bacterial pellet in 0.5 M $NaHCO_3$, pH 8.0 to a concentration of 2.5 x 10^9 colony-forming units/mL before addition of fluorescein (FITC; Invitrogen, Carlsbad, USA). Bacterial concentrations were approximated by optical density readings at 600 nm according to a standard curve for each strain used.

A FITC concentration of 0.15 mg/mL was used for staining *P. gingivalis* strain W83 and its isogenic mutant, and a concentration of 0.015 mg/mL for staining the ATCC 33277 strain and its isogenic mutant, as previously determined[19,20]. Of note, a lower FITC concentration is required to stain the ATCC 33277 strain, presumably due to the fact that this strain produces significantly more FITC-stainable fimbriae than the W83 strain. The tubes with bacteria and FITC were subsequently incubated in the dark for 30 min at RT in a tube rotator. The bacteria were pelleted at 7000 x g for 5 min, and the pellet was washed 3 times with PBS to remove unbound FITC. Finally, the bacteria were resuspended to the desired concentration in RPMI 1640/FBS 10%/2 mM L-glutamine. Neutrophils were infected for 90 min and THP-1 derived macrophages for 22 h with an MOI of 100, as described above.

To measure the bacterial internalization rate, the extracellular fluorescence (representing associated but not internalized bacteria) was quenched using 0.2% trypan blue (Thermo Fisher Scientific, Waltham, USA). Subsequently, two washing steps with PBS were performed to remove excessive trypan blue. Both quenched and non-quenched cell samples were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, USA) for 15 min prior to flow cytometric analyses.

An Accuri™ C6 Flow cytometer was used to measure the mean fluorescence intensity (MFI) of the FITC-positive cells. The association index of each *P. gingivalis* strain was calculated by multiplying the percentage of FITC-positive cells with associated bacteria (i.e. intracellular + extracellularly bound bacteria) with the MFI of these cells, divided by 100, as previously described[21]. The internalization index of each *P. gingivalis* strain was calculated by multiplying the percentage of cells with internalized bacteria (cells positive for FITC after trypan blue quenching) with the MFI of these cells, divided by 100 [21].

Immunofluorescence and confocal microscopy. Sterile coverslips were placed in each well of a 24-well plate. A total of 5 x 10^5 neutrophils, in 0.5 mL RPMI 1640 medium with 2mM L- glutamine and 10% autologous serum, were seeded into each well and allowed to settle for one hour at 37°C and 5% CO_2.

P. gingivalis infections were carried out as described above at a MOI of 100 for 90 min at 37°C and 5% CO2. After infection, the culture medium was removed and cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Tween. Blocking was performed with 1% bovine serum albumin. For the visualization of *P. gingivalis*, polyclonal rabbit antibodies raised against whole cells of *P. gingivalis* ATCC 33277 were used in combination with goat anti-rabbit Alexa Fluor 488-conjugated antibodies (Invitrogen, Eugene, USA). 4′,6-diamidino-2-phenylindole (DAPI) was used to stain DNA. Three washes with PBS were performed after each of the above steps following fixation of the cells. Images were recorded using a Leica TCS SP8 inverted confocal microscope (Leica Microsystems Inc., Buffalo Grove, USA).

LDS-PAGE. Lithium dodecyl sulphate (LDS)-PAGE was performed using 10% NuPAGE gels (Invitrogen, Carlsbad, USA). Protein concentrations of cell lysates were determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) and frozen at -20°C until further use. Equal amounts of protein samples were incubated with LDS sample buffer for 10 min at 95°C, separated by LDS-PAGE, and either stained with SimplyBlue™ SafeStain (Life Technologies, Carlsbad, USA) or processed further for Western blotting.

Western blotting. For Western blotting, proteins were transferred from the gel to a nitrocellulose membrane (Whatman, Buckinghamshire, UK) by semi-dry blotting (200 mA, 75 min). Upon transfer, the membrane was blocked overnight at 4°C with 5% skim milk (Oxoid, Basingstoke, UK) in PBS. Afterwards the blot was rinsed once with PBS-T to remove residual skim milk. Primary rabbit-anti-PPAD antibody[22] in PBS-T (1:2000) was added, and the blot was incubated for 3 h at RT. After removing the non-bound primary antibodies by 4 washes with PBS-T, the blot was incubated with IRDye 800CW goat-anti-rabbit antibody (LI-COR Biosciences, Lincoln, USA) in PBS-T (1:10,000) protected from light for 45 min. Lastly, background was reduced by washing 4 times with PBS-T and subsequently washing twice with PBS to remove the tween. Fluorescence was detected with the LI-COR ODYSSEY® (LI-COR Biosciences, Lincoln, USA) infrared imaging system.

Sample preparation for mass spectrometry. For SILAC experiments, THP-1 cells were cultured for 5-9 generations in RPMI 1640 (Silantes, Munich, Germany) with heavy arginine (Arg-6) and lysine (Lys-6). Cells were differentiated and lysed as described above, and incorporation of heavy amino acids was tested by mass spectrometry (MS) as described in detail below. Protein concentration was determined, and equal amounts of heavy and light THP-1 lysates were mixed.

Lysates of neutrophils and macrophages were processed for MS analyses as described before[23]. Briefly, proteins were bound to Strataclean resins (Agilent Technologies, Santa Clara, CA, USA) and subsequently alkylated, reduced and digested by trypsin. Resulting peptides were purified by C18 stage-tip purification and dried until further use.

Mass spectrometry of neutrophils. Purified peptides were analyzed by reversed phase liquid chromatography (LC) electrospray ionization (ESI) MS/MS using an Orbitrap Elite (Thermo Fisher Scientific, Waltham, USA). In brief, in-house self-packed nano-LC columns (20 cm) were used to perform LC with an EASY-nLC 1200 system (Thermo Fisher Scientific, Waltham, USA). The peptides were loaded with buffer A (0.1% acetic acid (v/v)) and subsequently eluted by a non-linear gradient from 1% to 99% buffer B (0.1% acetic acid (v/v), 94.9% acetonitrile) over a period of 156 min. A full scan was recorded in the Orbitrap with a resolution of 60,000. The twenty most abundant precursor ions were consecutively isolated in the linear ion trap and fragmented via collision-induced dissociation (CID). Unassigned charge states as well as singly charged ions were rejected and the lock mass option was enabled.

Database searching was done with Sorcerer-SEQUEST 4 (Sage-N Research, Milpitas, USA). After extraction from the raw files, *.dta files were searched with Sequest against a target-decoy database with a set of common laboratory contaminants. A database for the respective peptide/protein search was created from the published genome sequences of the W83 strain and the human genome, which were downloaded from Uniprot (http://www.uniprot.org) on 14/07/2016. The created database contained a total number of 148,472 proteins. Database search was based on a strict trypsin digestion with two missed cleavages permitted. No fixed modifications were considered. Oxidation of methionine, carbamidomethylation of cysteine and citrullination of arginine were considered as variable modifications. The mass tolerance for precursor ions was set to 10 ppm and the mass tolerance for fragment ions to 1 Da. Validation of MS/MS based peptide and protein identification was performed with Scaffold v.4 (Proteome Software, Portland, USA). A false discovery rate (FDR) of 0.1% was set for filtering the data. Protein identifications were accepted if at least 2 identified peptides were detected with the above-mentioned filter criteria in 2 out of 3 biological replicates. Protein data were exported from Scaffold and further curated in Microsoft Excel 2013 before further analysis. Possible citrullination of proteins was detected by a mass shift of 1 Dalton in arginine-containing peptides. To exclude false-positive identifications, peptides containing asparagine and/or glutamine were excluded from this analysis, because citrullination of arginine cannot be distinguished from deamidation of asparagine or glutamine.

Quantitative values of protein abundances in neutrophil samples were obtained by summing up all spectra associated with a specific protein within a sample, which includes also those spectra that are shared with other proteins. To allow comparisons, spectral counts were normalized by applying a scaling factor for each sample to each protein adjusting the values to normalized quantitative values.

Mass spectrometry of THP-1 macrophages. Purified peptides were analyzed by reversed phase LC ESI MS/MS using an Orbitrap Elite as described above for the neutrophil proteome samples. Database searching and SILAC quantification were done with MaxQuant version 1.5.7.0 [24]. The database search was done with the published genome sequences of the W83 strain and the human genome, which were downloaded from Uniprot (http://www.uniprot.org) on 14/07/2016. The MaxQuant generic Contaminants database was used. Database search was based on a strict trypsin digestion with two missed cleavages permitted. No fixed modifications were considered. Oxidation of methionine and

citrullination of arginine were considered as variable modifications. MaxQuant-computed H/L ratios were loaded into Perseus 1.5.8.5 [25] and filtered for "Potential contaminant", "Only identified by site", "Reverse" and for protein groups being identified with more than one unique peptide. The resulting list was imported into TMEV[26,27], and a one-way ANOVA was applied. Proteins with p-values ≤ 0.01 were considered significant. For stringent analysis of citrullination, peptides with a mass shift of 1 Dalton that contained asparagine or glutamine residues were eliminated.

Bioinformatic analyses. Heat maps were created using the TM4 Multi Experiment Viewer (TMEV)[26,27] stand-alone client version 4.8.1. Venn diagrams were created using a webtool provided by the University of Gent (http://bioinformatics.psb.ugent.be/webtools/Venn/). Principal component analyses (PCA) were performed on the spectral counts of the neutrophil and macrophage data using the FactoMiner package version 1.39 [28], and visualisation of the results was performed with the Plotly package version 4.7.1 [29]. Both packages were run on R version 3.4.2 [30]. Functional analysis of the proteome data was mainly done with the bioinformatic tool STRING[31] (https://string-db.org/), which visualizes protein-protein interactions based on direct (physical) and indirect (functional) interactions. The software also performs pathway enrichment analysis based on gene ontology (GO) terms.

Statistical analyses. All statistical analyses were performed with GraphPad Prism version 6 (GraphPad Software, La Jolla, USA). Two groups were compared by performing a paired, two-tailed student's t-test. Significance was defined as a p-value ≤ 0.05.

Medical Ethical Committee Approval. Blood donations from healthy volunteers were collected with approval of the medical ethics committee of the University Medical Center Groningen (UMCG; approval no. Metc2012-375). All blood donations were obtained after written informed consent from all volunteers, and adhering to the Helsinki Guidelines.

Biological and chemical safety. *P. gingivalis* was handled following appropriate safety and containment procedures for biosafety level 2 microbiological agents. All experiments involving human cells were performed under appropriate safety conditions. All chemicals and reagents applied in this study were handled according to local guidelines for safe usage and protection of the environment.

Data availability. The mass spectrometry data are deposited in the ProteomeXchange repository PRIDE (https://www.ebi.ac.uk/pride/). The dataset identifier for the neutrophil experiments is PXD009107 (Username for review: reviewer21944@ebi.ac.uk; Password: wA0Bqm1q) and the dataset identifier for the macrophage experiments is PXD011003 (Username for review: reviewer57936@ebi.ac.uk; Password: WJupSEOy).

Results

PPAD modulates the human neutrophil proteome upon *P. gingivalis* internalization

Previous studies have shown that the virulence of different *P. gingivalis* isolates may differ quite substantially. In particular, this was shown for the *P. gingivalis* type strains W83 and ATCC 33277 [12,20,32]. Therefore, prior to assessing the impact of these type strains and the respective PPAD proteins on human neutrophils, we first assessed their association with and internalization by these professional phagocytes, and the same was done for the respective PPAD-deficient bacteria. Interestingly, we measured about 100-fold higher association and internalization indices, and three-fold higher infection coverages for the ATCC 33277 wild-type and PPAD-deficient strains than for the W83 wild-type and PPAD-deficient strains (Fig. 1a, Fig. S1a). This difference was reflected in immunofluorescence microscopy analyses, where substantially higher numbers of neutrophil-adherent and -internalized bacteria of the ATCC 33277 strain were observed compared to the W83 strain (Fig. 1b). In addition, a significant difference in the association and internalization of the W83 wild-type and PPAD-deficient strains was observed where, in accordance with our previous observations, the bacteria lacking PPAD were phagocytosed more effectively than the wild-type cells[15]. Such a difference was not detectable for the wild-type and PPAD-deficient ATCC 33277 strains. Of note, these findings were complemented by Western blotting of the neutrophil lysates, showing that the neutrophils infected with PPAD-proficient bacteria did indeed contain PPAD, whereas PPAD was absent from neutrophils infected with PPAD-deficient bacteria (Fig. 1c).

Based on the different association and internalization behavior of the *P. gingivalis* W83 and ATCC 33277 strains, we decided to investigate the impact of exposure of human neutrophils to either of these two type strains, or the respective PPAD-deficient strains by proteomics. Accordingly, samples from infected neutrophils were used for MS analyses, and samples from non-infected neutrophils were used as a control. Altogether, 281 proteins were identified across the different conditions (Table S1). Only 97 of these proteins were identified in all samples, highlighting substantial differences between the individual infection conditions. The heat map in Figure S2 visualizes the normalized quantities of these 97 consistently identified proteins under the different conditions. Notably, the non-infected control neutrophils showed a quantitative protein fingerprint that is clearly distinct from those of the infected neutrophils. In addition, the neutrophils infected with the PPAD-deficient *P. gingivalis* cells showed protein fingerprints that are distinct from the neutrophils infected with wild-type *P. gingivalis*. A subsequent principal component analysis (PCA) of the data revealed that the proteome of the non-infected control neutrophils is substantially different from the proteomes of neutrophils that have been exposed to *P. gingivalis* (Fig. 2). This difference can be mainly seen in the second principal component, which explains 26% of the difference in protein abundances. Further, the PCA showed that deleting the PPAD gene in the W83 strain has only a relatively minor effect on the abundance of proteins of infected neutrophils. The neutrophils infected with PPAD-proficient or PPAD-deficient cells of strain W83 cluster

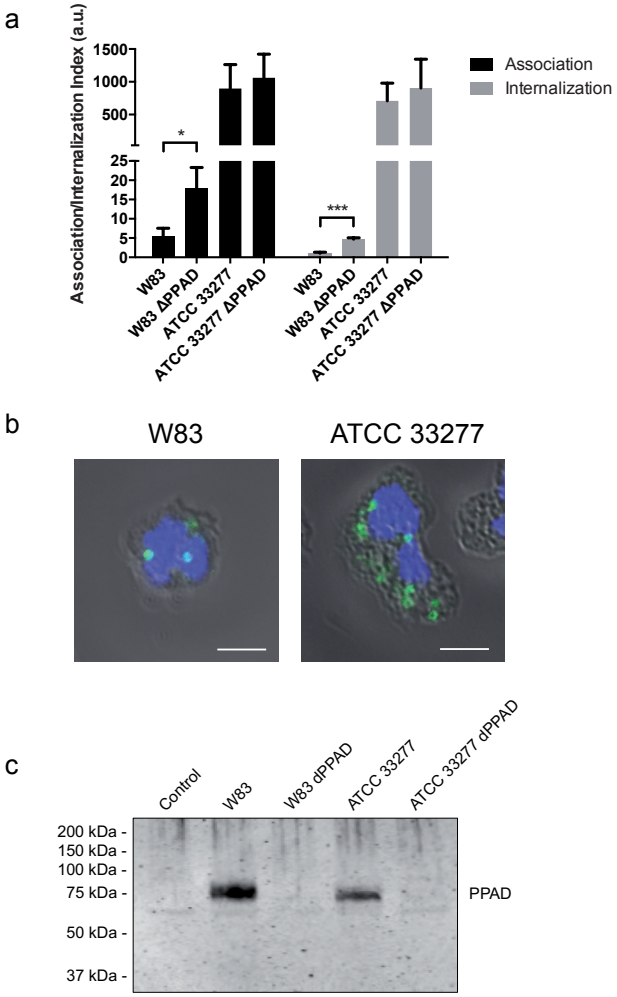


Figure 1: Phagocytosis of PPAD-proficient and -deficient *P. gingivalis* strains W83 and ATCC 33277 by human neutrophils. a, The differential neutrophil association and internalization behavior of the investigated *P. gingivalis* strains ATCC 33277 and W83, and their isogenic PPAD-deficient mutants was quantified by FITC labeling of the bacteria and subsequent flow cytometry. Association refers to all bound and intracellular bacteria, while internalization refers to intracellular bacteria only. **b**, Representative images of *P. gingivalis* bound to or internalized by human neutrophils visualized by confocal microscopy; green, immuno-labeled *P. gingivalis*, blue: DAPI-stained DNA; scale bars, 5 µm. **c,** Western blot detection of PPAD in neutrophil lysates upon infection with the investigated *P. gingivalis* strains. PPAD has an apparent molecular mass of ~75 kDa, which corresponds to the A-lipopolysaccharide-modified form of the PPAD protein [47]. Of note, the neutrophil lysates used for the presented Western blot were also used for the proteomics analyses. Data in (**a**) are presented as mean values ± the standard deviation (SD). Specifically, they represent the means of experiments with neutrophils from three different healthy donors; for each infection experiment four replicates were performed. Statistical significance was assessed using a two-tailed unpaired Student's *t*-tests. **P*<0.05, ****P*<0.001.

very closely together, while the absence of PPAD from cells of the ATCC 33277 strain leads to a major shift in the relative abundance of neutrophil proteins. The latter is observed mainly in the third principal component, which explains 16% of the difference in protein abundances (Fig. 2). These observations show that PPAD production by the ATCC 33277 strain has a much higher impact on the relative protein quantities in neutrophils than PPAD production by the W83 strain. This difference probably relates to the fact that PPAD-proficient and -deficient cells of the ATCC 33277 strain are much more effectively internalized by the neutrophils (Fig. S1a).

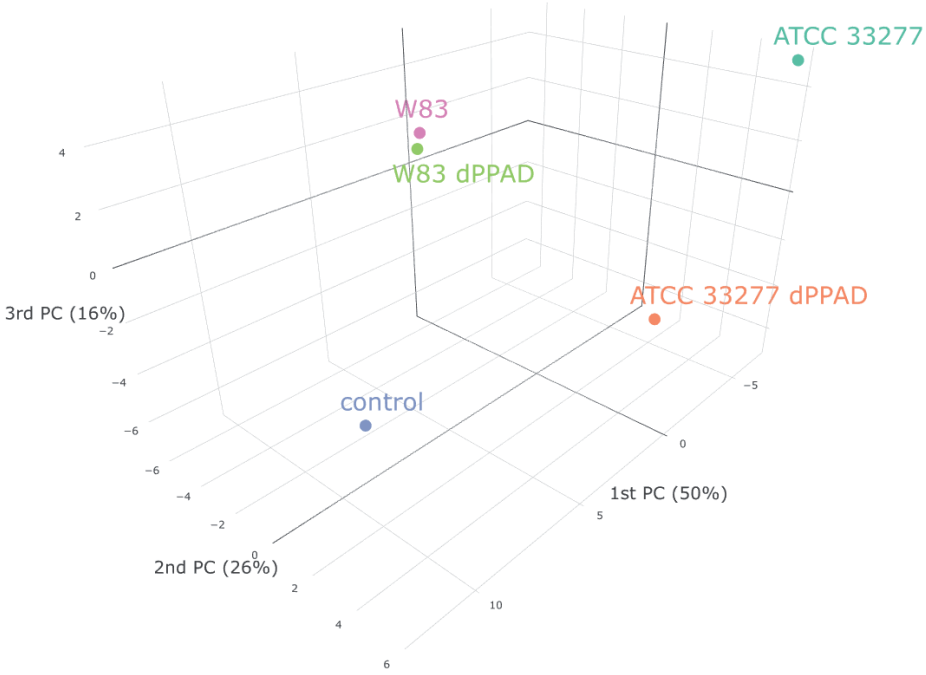


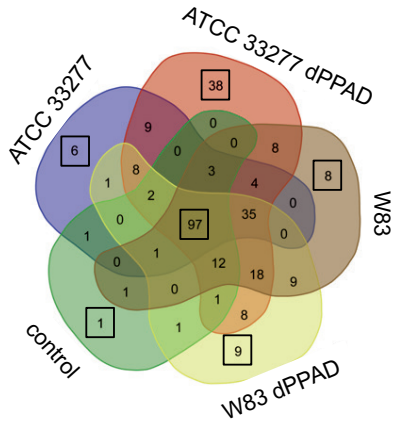
Figure 2: Differential impact of *P. gingivalis* ATCC 33277 and W83 on the neutrophil proteome. Principal component analysis (PCA) was performed using spectral counts obtained for consistently identified proteins detected in the different infection conditions and the uninfected control. The first principal component (PC) explains 50%, the second PC 26% and the third PC 16% of the differences. Note that: (i) the non-infected control sample clusters far away from all infected neutrophil samples on the first PC; (ii) differences in samples of neutrophils infected with the W83 or ATCC 33277 strains are mainly explained by the second PC; (iii) neutrophil samples infected with the W83 wild-type or its PPAD-deficient derivative cluster closely together; and (iv) neutrophil samples infected with the ATCC 33277 wild-type or its PPAD-deficient derivative are separated on the third PC.

PPAD interferes with antimicrobial defense systems of neutrophils

Since the strongest effects of a PPAD deletion on the abundance of neutrophil proteins were observed for the ATCC 33277 strain, we focused further analyses on this strain. Neutrophils infected with the PPAD-deficient ATCC 33277 strain showed 38 unique proteins that were not identified in any of the other infection settings or the uninfected control, making it a very distinct condition (Fig. 3a). A direct comparison of neutrophils infected with the ATCC 33277 wild-type or its PPAD mutant identified six proteins to be less abundant and four proteins to be more abundant when PPAD was present (Fig. 3b). Of interest, the human PAD4 enzyme was detected at higher levels in the presence of PPAD, which is indicative of higher human PAD-activities in the presence of the bacterial PPAD enzyme. On the other hand, two *P. gingivalis* proteins and four human proteins were found to be significantly less abundant in the presence of PPAD. Specifically, this concerned the receptor antigen B (B2RHG8_PORG3) and the arginine-specific gingipain RgpA (B2RM93_PORG3) of *P. gingivalis*, and the antimicrobial azurocidin (CAP7_HUMAN), the proteasome subunit alpha-1 (PSA1_HUMAN), the elongation factor 1-gamma (EF1G_HUMAN) and the spectrin alpha chain (A0A0D9SF54_HUMAN) of the neutrophils.

Neutrophils infected with the PPAD-proficient or -deficient ATCC 33277 strains shared the presence of 157 proteins, while 9 unique proteins were identified when PPAD was present and 85 unique proteins when PPAD was absent (Fig. 4a). While the defensin 4 (DEFA4), the defensin 6 (DEF6) and the vesicle-related protein RAB11 (RAB11A) were found in the presence of PPAD, the majority of host defense proteins were only found in the absence of PPAD. Of note, a functional enrichment shows that most of the uniquely identified proteins upon infection with the PPAD-deficient strain are related to immune response-activating cell surface receptor signaling pathways and antigen processing and presentation (Fig. 4b). Some examples of these proteins are the actin-related protein 2/3 complex (ARPC3) involved in phagocytosis and the neutrophil cytosol factor 4 (NCF4) involved in phagocytosis and oxidative burst (Fig. 4a). The main host defense factors shown to be detectable only in the absence of PPAD are the proteasome subunits alpha and beta (encircled in Fig. 4a), which are involved both in immune response activation and antigen processing and presentation. Also, some positive regulators of host defense responses were only detected in the absence of PPAD. These include the calcium-binding protein S100A12 and the MAP kinase ERK2 (MAPK1). A detailed inspection of the impact of PPAD on the neutrophil proteasome revealed that nine out of twelve identified proteasome-related proteins are apparently less abundant when PPAD is present (Fig. 4c). Interestingly, only two of these twelve proteins were identified in the uninfected control neutrophils (i.e. PSMA5 and PSMA6), suggesting an up-regulation of the proteasome upon infection, especially with PPAD-deficient *P. gingivalis*.

a



Total number of identified proteins				
ATCC 33277	ATCC 33277 dPPAD	W83	W83 dPPAD	Ctrl
166	242	195	201	119

b

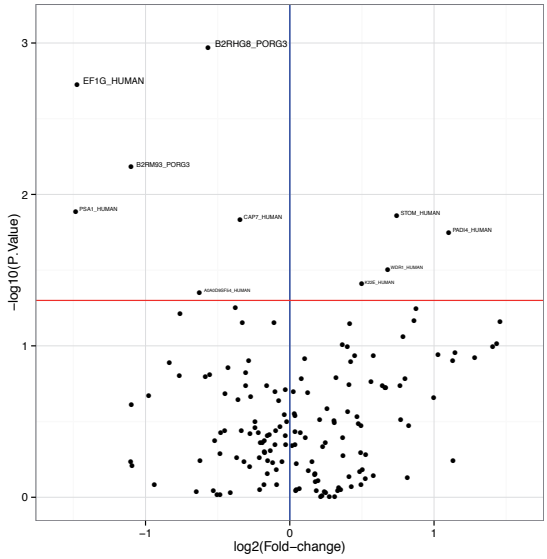
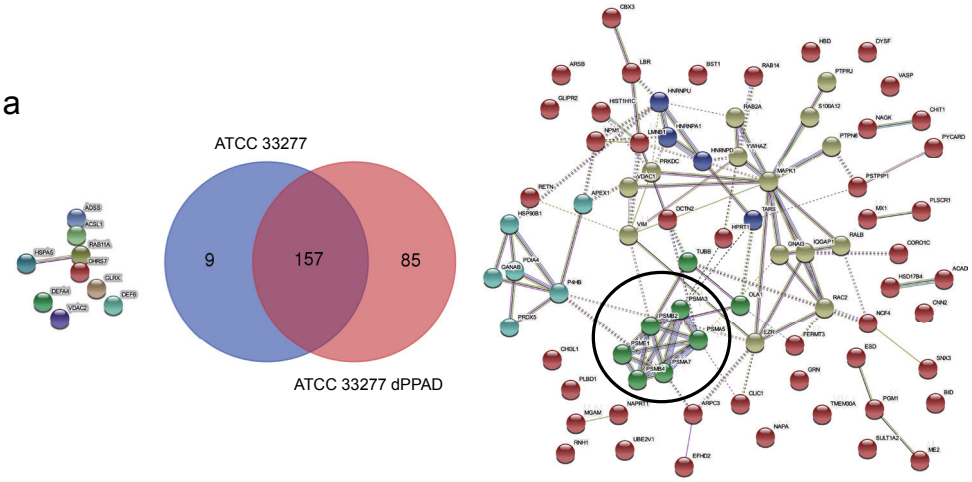


Figure 3: Differentially expressed proteins in neutrophils infected with PPAD-proficient or –deficient *P. gingivalis* ATCC 33277. **a**, Venn diagram showing the overlap in identified neutrophil proteins in the different infection conditions and the uninfected control. The Table specifies the total number of identified proteins per sample. **b**, Volcano plot showing the differentially expressed neutrophil proteins infected with PPAD-proficient or -deficient *P. gingivalis* ATCC 33277. The x-axis shows the log2 fold-change and the y-axis the log10 p-value. Each dot represents a single protein. Proteins above the red line are significantly up- or down-regulated, and they are labelled with the respective Uniprot identifier.

a



b

#pathway ID	pathway description	observed gene count	false discovery rate
GO.0002429	immune response-activating cell surface receptor signaling pathway	11	5.03E-06
GO.0002479	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	7	5.03E-06
GO.0006915	apoptotic process	19	5.03E-06
GO.0031349	positive regulation of defense response	12	5.03E-06
GO.0042770	signal transduction in response to DNA damage	8	5.03E-06

c

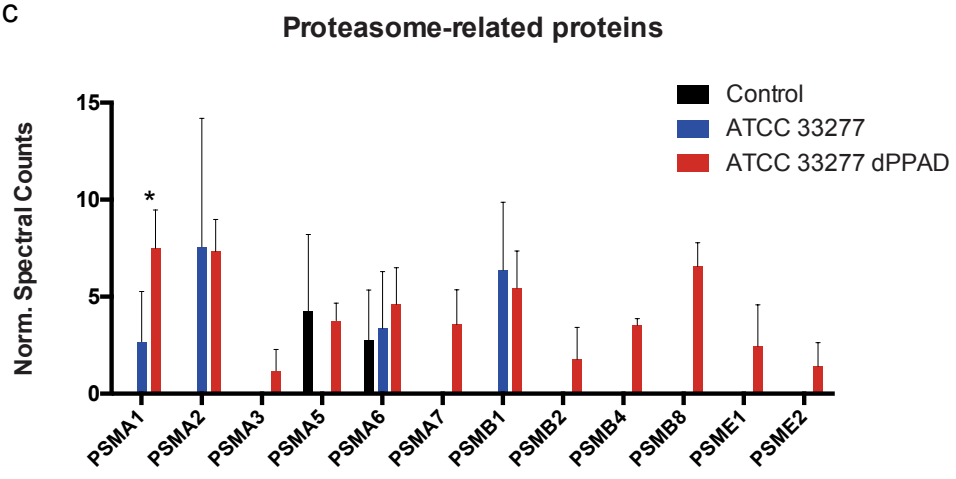


Figure 4 : PPAD-specific downregulation of neutrophil defense mechanisms. a, Venn diagram and STRING networks illustrating the unique proteins identified in neutrophils infected with PPAD-proficient or -deficient *P. gingivalis* ATCC 33277. Proteins in the STRING networks are labelled with the respective gene names. The black circle in the network on the right marks a cluster of proteasome-related proteins. Connecting lines between proteins and color codes are as defined by STRING (http://version10.string-db.org/help/getting_started/). **b**, Pathway enrichment based on gene ontology (GO) identifiers of neutrophil proteins uniquely identified upon infection with PPAD-deficient *P. gingivalis* ATCC 33277. Note that these proteins are part of the STRING network in Figure 5a. **c**, Normalized spectral counts measured for twelve proteasome-related neutrophil proteins in the different infection conditions and the uninfected control. Data are shown for the alpha-proteasome subunits (PSMA) 1, 2, 3, 5, 6 and 7, the beta-proteasome subunits (PSMB) 1, 2, 4 and 8, and the two proteasome activator complex subunits (PSME) 1 and 2.

Citrullination of major neutrophil host defense proteins in the presence of PPAD

Since PPAD has citrullinating activity on human proteins[13], we determined which proteins are targets of citrullination in our neutrophil protein dataset. To this end, we first inspected all proteins from infected and uninfected neutrophils, so that both the citrullination by human PAD enzymes and PPAD-specific citrullination would be captured. This led to the identification of 34 citrullinated proteins in total, which are listed in Table 1. Of note, a relatively small number of proteins was identified to be citrullinated in neutrophils infected with the wild-type ATCC 3327 strain, which relates to the overall lower number of identified proteins compared to neutrophils infected with the PPAD-deficient ATCC 33277 strain (Fig. 3a). In lysates from W83-infected neutrophils, we detected significantly more citrullinated proteins and, in addition, we observed substantial differences in the number of citrullinated proteins from neutrophils infected with the PPAD-proficient or -deficient W83 strains (Table 1). These results motivated us to also assess the citrullination of neutrophil proteins upon infection with *P. gingivalis* W83, although this strain is internalized at much lower levels than the ATCC 33277 strain (Fig. 1a, Fig. S1a).

Several major host defense proteins were subject to citrullination (Table 1), including the integrin alpha-M, proteinase 3, myeloperoxidase and the proteasome subunit alpha type-6. While 13 of these proteins were citrullinated also in the absence of PPAD, 20 neutrophil proteins were found to be citrullinated only in the presence of PPAD (Table 1, Fig. 5). Two of the proteins that were only identified as citrullinated in neutrophils infected with PPAD-proficient bacteria were proteinase 3 and cathepsin G. Of note, these two central neutrophil proteases have both been implicated in the pathogenesis of RA[33,34]. Lastly, we identified two citrullinated *P. gingivalis* proteins, namely the two gingipains RgpA and Kgp (Table 1), which is consistent with our previous observation that these bacterial proteins are also targets of citrullination[12]. However, RgpA and Kgp were also found to be citrullinated in the absence of PPAD, showing that these proteins are also targets for PAD enzymes from the neutrophils.

Table 1: Overview of citrullinated proteins. A total of 32 human and 2 *P. gingivalis* proteins in the present dataset was identified as being citrullinated. Citrullinated peptides were filtered for the absence of asparagine and glutamine residues that could potentially be deamidated. The Table shows for each protein the Uniprot identifier, protein description and molecular weight. An orange rectangle indicates the detection of the protein in at least one control condition; a red rectangle indicates the unique detection of the protein in either one or two of the conditions with PPAD being present; a white square indicates that no high-confidence citrullinated peptides were detected.

Uniprot identifier	description	MW (Da)	neutrophils only	W83	W83 dPPAD	ATCC 33277	ATCC 33277 dPPAD
PERM_HUMAN	Myeloperoxidase	83,869					
ACTG_HUMAN	Actin, cytoplasmic 2	41,793					
PRDX2_HUMAN	Peroxiredoxin-2	21,892					
ITAM_HUMAN	Integrin alpha-M	127,179					
GDIR2_HUMAN	Rho GDP-dissociation inhibitor 2	22,988					
CHIT1_HUMAN	Chitotriosidase-1	51,681					
FLNA_HUMAN	Filamin-A	280,739					
PDIA3_HUMAN	Protein disulfide-isomerase A3	56,782					
CATG_HUMAN	Cathepsin G	28,837					
FABP5_HUMAN	Fatty acid-binding protein 5	15,164					
HORN_HUMAN	Hornerin	282,390					
TKT_HUMAN	Transketolase	67,878					
HBA_HUMAN	Hemoglobin subunit alpha	15,258					
ALBU_HUMAN	Serum albumin	69,367					
ANXA1_HUMAN	Annexin A1	38,714					
DESP_HUMAN	Desmoplakin	331,774					
PERE_HUMAN	Eosinophil peroxidase	81,040					
DSG1_HUMAN	Desmoglein-1	113,748					
TRFL_HUMAN	Lactotransferrin	78,182					
DSC1_HUMAN	Desmocollin-1	99,987					
MOES_HUMAN	Moesin	67,820					
PRTN3_HUMAN	Proteinase 3 (Myeloblastin)	27,807					
APEX1_HUMAN	DNA-(apurinic or apyrimidinic site) lyase	35,555					
DPEP3_HUMAN	Dipeptidase 3	53,687					
PSA6_HUMAN	Proteasome subunit alpha type-6	27,399					
CH3L1_HUMAN	Chitinase-3-like protein 1	42,625					
HS90A_HUMAN	Heat shock protein HSP 90-alpha	84,660					
CD97_HUMAN	CD97 antigen	91,869					
TLN1_HUMAN	Talin-1	269,767					
ECP_HUMAN	Eosinophil cationic protein	18,385					
A0A0A6YYJ9_HUMAN	Solute carrier organic anion transporter family member	82,544					
VAT1_HUMAN	Synaptic vesicle membrane protein VAT-1	41,920					
CPG1_PORG3	Gingipain R1	185,325					
KGP_PORG3	Lys-gingipain	187,262					

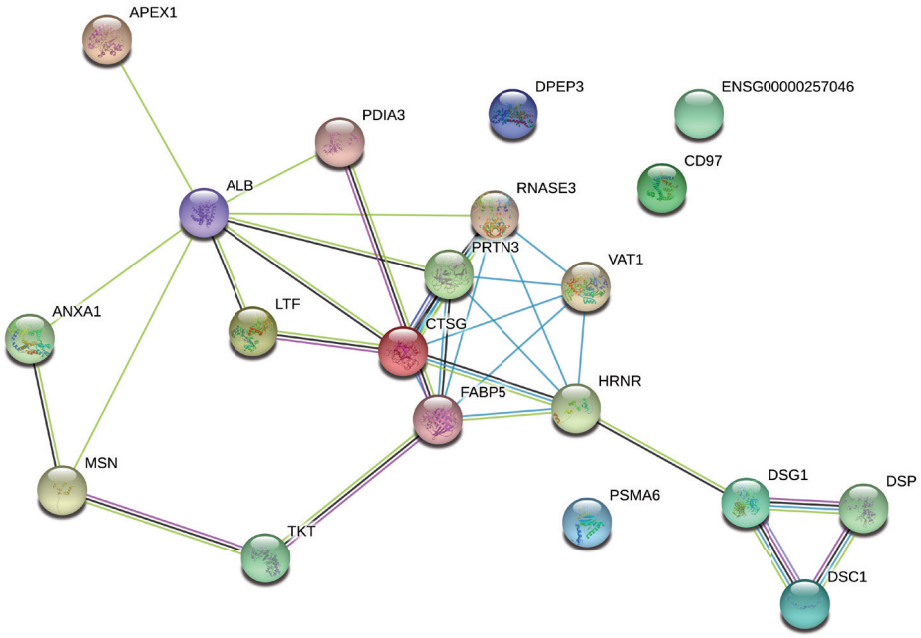


Figure 5: Citrullination of host defense proteins in neutrophils. STRING network analysis of 20 proteins that were unambiguously citrullinated in neutrophils infected with PPAD-proficient bacteria. Only proteins that were identified uniquely in the presence of PPAD-proficient W83 and/or ATCC 33277 are shown. All potentially citrullinated peptides containing asparagine or glutamine residues were excluded in this analysis, because they could lead to false-positive identifications due to possible deamidation.

PPAD modulates the proteome of human macrophages to a lesser extent than the neutrophil proteome

Macrophages are the second type of innate immune cells implicated in periodontitis and RA. Therefore, we also examined the impact of *P. gingivalis* and PPAD on the proteome of human macrophages, using a similar experimental set-up as for the experiments with neutrophils described above. However, by applying a macrophage cell line, we were able to implement a SILAC approach to obtain fully quantitative data. As shown in Figure 6, the ATCC 33277 strain showed higher association and internalization indices for macrophages than the W83 strain, which is consistent with our observations upon neutrophil infection (Fig. 1a). However, PPAD does not seem to influence the internalization of strain W83 in macrophages, and PPAD-proficiency leads to higher attachment and internalization indices in the case of the ATCC 33277 strain (Fig. 6). This implies that PPAD could act on surface proteins of macrophages to influence the attachment behavior. PCA analysis of the MS data from macrophages infected with *P. gingivalis* showed that infection drastically changed the macrophage proteome compared to the uninfected control samples (Fig. 7A). However, in the case of macrophages, the differences observed

upon infection with PPAD-proficient or -deficient strains (Fig. 7a, b) were different from those observed in the equivalent neutrophil infection experiments (Figs. 2 and 3). In particular, the impact of PPAD proficiency on the macrophage proteome was more extensive for the W83 strain than the ATCC 33277 strain (Fig. 7a). Global analysis of the whole dataset showed that 276 macrophage proteins were expressed in all situations (Table S2), while much lower numbers of unique macrophage proteins were identified under the different infection conditions. Of note, 42 unique proteins were identified in uninfected control macrophages, which makes this condition more diverse than the infected macrophage conditions.

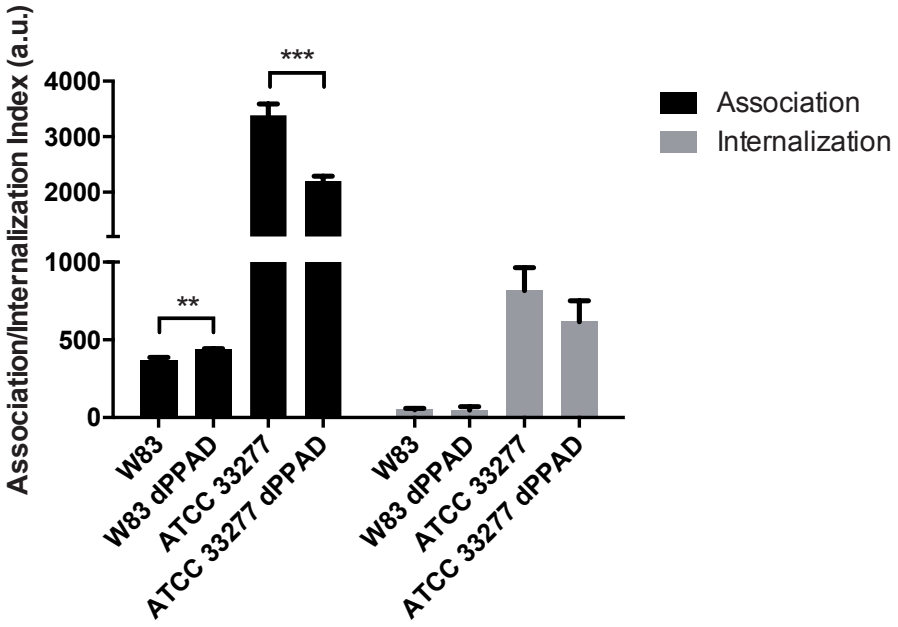
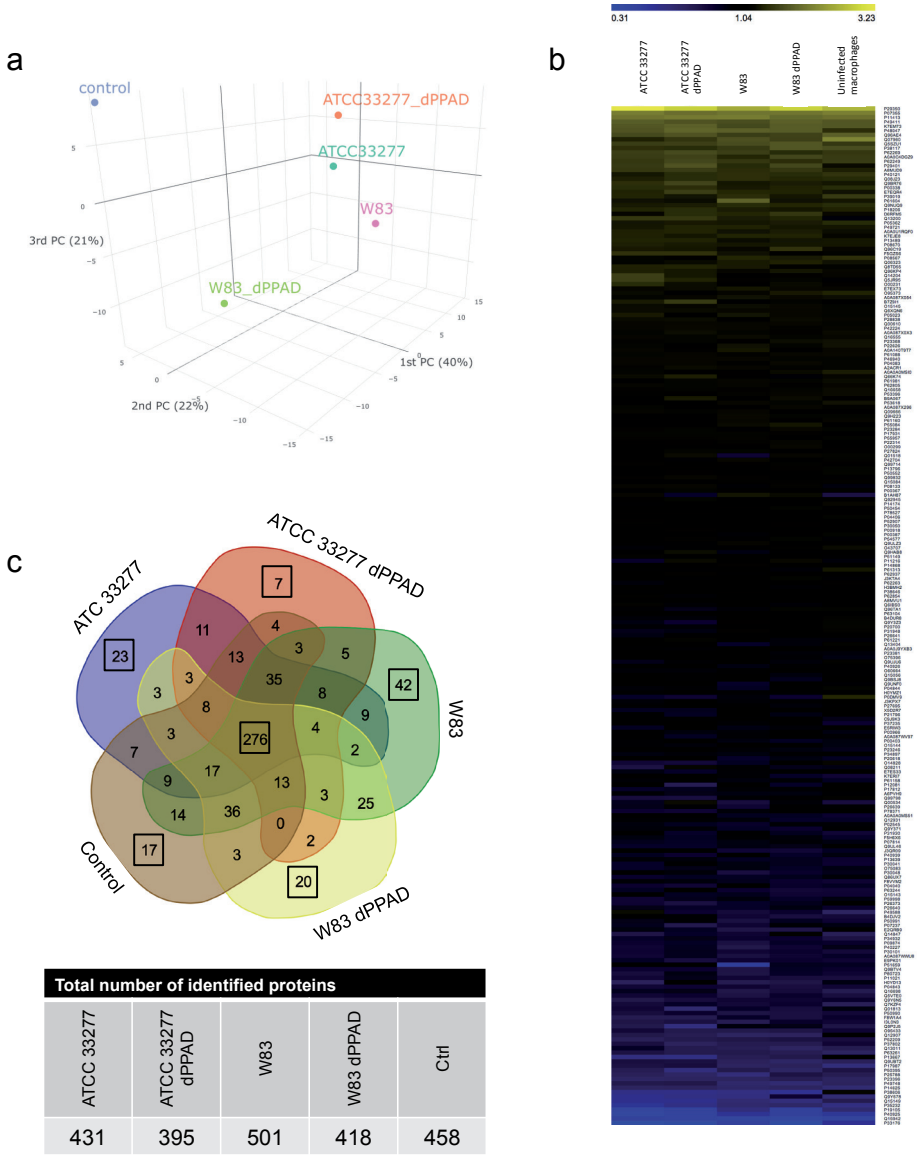


Figure 6: Phagocytosis of PPAD-proficient and -deficient *P. gingivalis* strains W83 and ATCC 33277 by human macrophages. The differential THP1 macrophage association and internalization behavior of the investigated *P. gingivalis* strains ATCC 33277 and W83, and their isogenic PPAD-deficient mutants was quantified by FITC labeling of the bacteria and subsequent flow cytometry. Association refers to all bound and intracellular bacteria, while internalization refers to intracellular bacteria only. Data are the mean values of three replicates (±SD). Statistical significance was assessed using a two-tailed unpaired Student's *t*-tests. ***P*<0.01, ****P*<0.001.



Total number of identified proteins				
ATCC 33277	ATCC 33277 dPPAD	W83	W83 dPPAD	Ctrl
431	395	501	418	458

Figure 7: Strain-specific and PPAD-dependent impact of *P. gingivalis* on individual macrophage proteins. a, PCA of the three principal components (PC) explaining differences in the THP1 macrophage proteome observed for different infection conditions and the uninfected control. The first PC explains 40%, the second PC 22% and the third PC 21% of the differences. Note that (i) non-infected control macrophage samples cluster far away from all infection conditions; (ii) the macrophage samples from infections with different W83 and ATCC 33277 strains cluster apart; (iii) differences in the macrophage samples from infections with the PPAD-proficient or -deficient W83 strains are separated by the second PC; and (iv) differences in the macrophage samples from infections with the PPAD-proficient or -deficient ATCC 33277 strains are separated by the third PC. **b**, Heat map illustrating the relative

abundance of the identified macrophage proteins, based on heavy-light ratios. The heat map includes data for 276 proteins identified in all conditions. The y-axis specifies the individual proteins, and the x-axis marks the different infection conditions with *P. gingivalis* isolates and the uninfected control neutrophils. The colors indicate the relative abundance of the proteins: yellow indicates low abundance, black moderate abundance and blue high abundance. **c**, Venn diagram showing the overlap in identified macrophage proteins in the different infection conditions and the uninfected control. The table shows the total number of identified proteins per sample.

An in-depth analysis of the impact of PPAD shows that the amounts of ten proteins were significantly different when macrophages were infected with the PPAD-proficient or -deficient ATCC 33277 strains (Fig. 8a), and 17 proteins when macrophages were infected with the PPAD-proficient or -deficient W83 strains (Fig. 8b). Of interest, these proteins include the central immune regulator 'Signal transducer and activator of transcription' 1 (STAT1), the three phagocytosis-related proteins 'actin-related protein complex 2/3' (ARPC3), the 'cell division control' protein 42' (CDC42) and annexin A2 (ANXA2), the two proteasome proteins 'proteasome subunit alpha type-1' (PSMA1) and the 26S proteasome regulator (PSMD2), as well as the established RA autoantigen alpha-enolase (ENO1) (Fig. 9). Citrullination was unambiguously detected in only one protein, namely the myocyte enhancer factor-2c that is involved in the regulation of transcription. Citrullination of this protein was mainly detected in macrophages infected with the ATCC 33277 wild-type strain, but also in macrophages infected with the ATCC 33277 and W83 PPAD-deficient bacteria, as well as in the uninfected control macrophages. This identification of only one citrullinated protein in the macrophages can probably be explained by the strict filter criteria that were used for the analysis where we excluded all potentially citrullinated peptides with asparagine and/or glutamine residues.

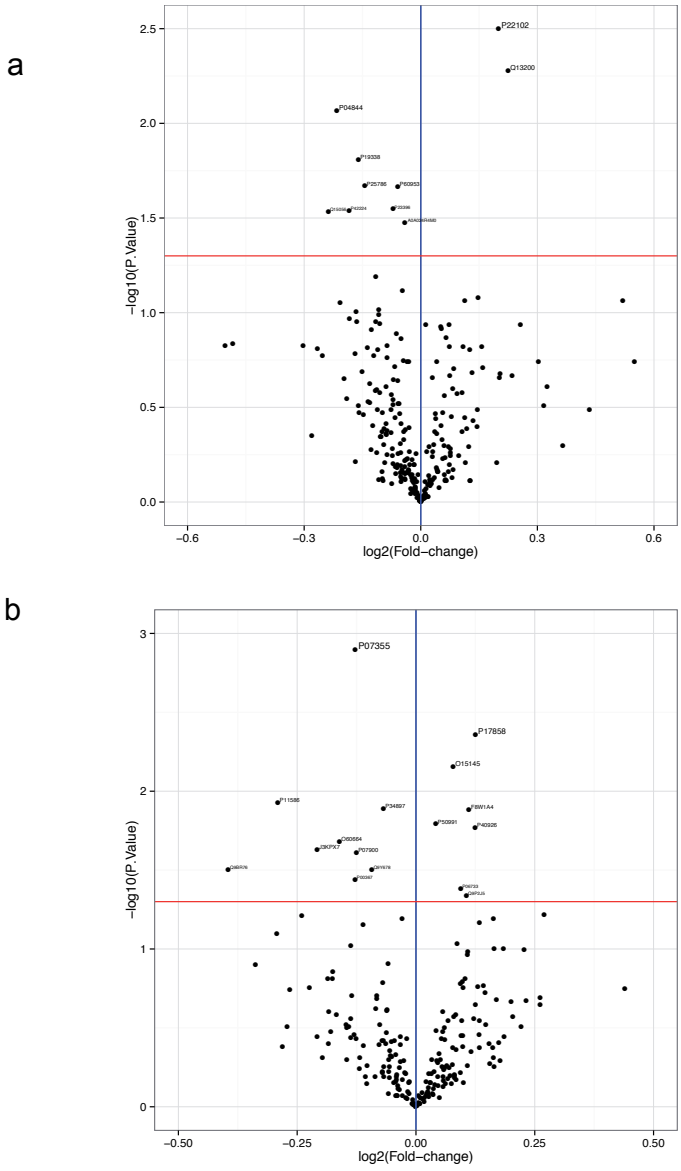


Figure 8: Differentially expressed proteins in macrophages infected with PPAD-proficient or -deficient *P. gingivalis* ATCC 33277 and W83. Volcano plot showing differentially expressed THP1 macrophage proteins upon infection with PPAD-proficient or -deficient *P. gingivalis* ATCC 33277 (**a**) or W83 (**b**). The x-axes show the log2 fold-changes and the y-axes the log10 p-values. Each dot represents a single protein. Proteins above the red line are significantly up- or down-regulated, and they are labelled with the respective Uniprot identifier.

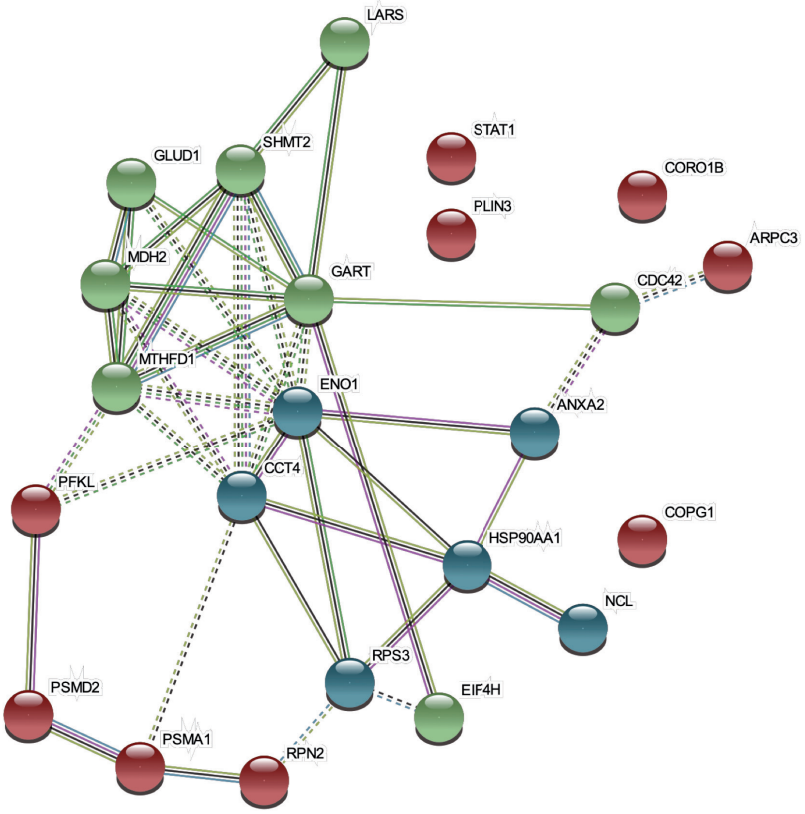


Figure 9: Network analysis of identified macrophage proteins. STRING network analysis of the THP1 macrophage proteins with significantly altered abundance upon infection with PPAD-proficient or -deficient *P. gingivalis* ATCC 33277 and W83 strains as identified in Figure 8.

Discussion

Here we present a first global study on the effects of the periodontal pathogen *P. gingivalis* and its secreted peptidylarginine deiminase PPAD on proteostasis and citrullination in human innate immune cells. In particular, we show that infection of human neutrophils and macrophages with *P. gingivalis* elicits drastic shifts in the proteome of these phagocytes (i.e. the 'immunome') in a strain-specific manner. Moreover, we show that the presence of PPAD has a substantial impact on particular immunome changes.

The present findings show that the *P. gingivalis* strain ATCC 33277 is phagocytosed more effectively by neutrophils and macrophages than the W83 strain. This matches well with previously documented observations that the virulence of these two widely studied *P. gingivalis* strains differs substantially, at least in animal infection models[32,35]. The difference in virulence can be related to

strain-specific proteome differences and differences in encapsulation. In particular, the ATCC 33277 strain is an abundant producer of 'major fimbriae', whereas these host cell-adhesive bacterial surface structures are absent from the W83 strain[12,36]. The latter difference would be sufficient to explain the presently observed differential behavior of these strains in terms of adhesion to and internalization by neutrophils and macrophages. In fact, the importance of major fimbriae for phagocytosis of *P. gingivalis* by macrophages was previously demonstrated[37,38]. It thus seems likely that the differential production of fimbriae is also, at least partially, responsible for differential phagocytosis of the ATCC 33277 and W83 strains by neutrophils as reported here. The escape of the W83 strain from neutrophils and macrophages is, most likely, further enhanced by the formation of a polysaccharide capsule, which is absent from the ATCC 33277 strain[6,39]. Together, these previously documented findings explain why the non-fimbriated and highly encapsulated W83 strain is more successful in evading professional phagocytes than the ATCC 33277 strain.

While PPAD is increasingly considered as a virulence factor of *P. gingivalis*, its role in the actual infective process is still relatively poorly defined. In the present study, we observed that in the ATCC 33277 strain, the ability to produce PPAD has a much more profound impact on the proteome of infected human neutrophils than is the case in the W83 strain. This difference suggests that, by more effectively evading phagocytosis, the W83 strain is less capable of modifying the neutrophil proteome than the ATCC 33277 strain. Conversely, PPAD has a more significant impact on neutrophil association and invasion by the W83 strain, which may be overshadowed in case of the ATCC 33277 strain by the high rate of phagocytosis. In turn, these observations imply a critical role of PPAD in neutrophils that have internalized high numbers of *P. gingivalis*. Of note, the effects of PPAD production by the W83 strain on the macrophage proteome appear stronger than those observed for the ATCC 33277 strain, albeit that the overall observed differences are less pronounced in macrophages than in neutrophils. It is tempting to speculate that these differences relate to the fact that in its natural niche, the periodontium, *P. gingivalis* is primarily challenged by neutrophils and subsequently by macrophages[17]. Hence, the immune evasive mechanisms of this pathogen may be more specifically targeted towards neutrophils than macrophages. This view would be consistent with the present finding that 20 neutrophil proteins were PPAD-specifically citrullinated while no PPAD-specific citrullination was observed for identified macrophage proteins. Of note, the fact that we identified 33 more unambiguously citrullinated proteins in neutrophils than in macrophages may suggest that the neutrophil proteome is more susceptible to this post-translational modification.

In the present study, analysis of the proteome of neutrophils infected with PPAD-proficient or -deficient *P. gingivalis* showed that the most pronounced differences relate to host defense proteins. Antimicrobials, defensins, phagocytosis-related proteins and immune-regulatory proteins were significantly less abundant or even undetectable upon infection with PPAD-proficient bacteria. This is in agreement with the notion that citrullination of proteins changes their charge and three-dimensional structure, which could in turn lead to an altered folding state or even unfolding[40]. Especially unfolding makes proteins more susceptible to proteolysis, which would explain the reduced abundance or absence of particular proteins. Clearly, proteases are abundantly present in neutrophils and, on top of

this, *P. gingivalis* produces several secreted gingipains. Conversely, the aggressive oxidative neutrophil environment will cause protein damage, which might make neutrophil proteins more susceptible to citrullination, either by the neutrophil PADs, by PPAD or both. In line with our present observations relating to the evasion of immune defenses by *P. gingivalis*, Bielecka and colleagues showed that PPAD is able to citrullinate parts of the complement system, leading to decreased chemotaxis of human neutrophils[14]. Moreover, we have recently shown that PPAD can literally neutralize the cationic antimicrobial peptide LP9, and help *P. gingivalis* to escape from NETs by histone citrullination[15]. On the other hand, PPAD does not seem to citrullinate or impair the function of human chemokines produced by macrophages, as shown by Moelants and colleagues[41]. Instead, they showed that chemokines are degraded by the secreted gingipains of *P. gingivalis*. Gingipains were also shown to impair the complement system and toll-like receptor (TLR) signaling in a recent study by Maekawa and colleagues[42]. Thus, it seems that the concerted action of PPAD and gingipains leads to corruption of the human innate immune system. Of note, our previous investigation on the impact of PPAD on bacterial proteins showed that this enzyme also citrullinates gingipains, which could modulate their proteolytic activity and protect them against self-cleavage or cleavage by other proteases[12].

The human proteasome has been implicated in several immune regulatory processes, as well as in antigen processing and presentation[43,44]. In addition, a functional proteasome was previously shown to be crucial for the destruction of intracellular pathogens[45,46]. Infection of neutrophils with *P. gingivalis* seems to up-regulate the proteasome machinery, but only when the bacteria cannot produce PPAD. This implies that PPAD-proficient bacteria can preclude proteasome-mediated destruction in neutrophils, which would be a new function of PPAD in immune evasion.

Lastly, the two central neutrophil proteins proteinase 3 and cathepsin G have been implicated in the pathogenesis of RA[33,34]. In our present study, we show for the first time that human proteinase 3 and cathepsin G are PPAD-dependently citrullinated in human neutrophils. The citrullination of these two proteins in neutrophils might thus be among the first steps in the development of ACPAs and the loss of tolerance to citrullination in RA. Altogether, our present observations place the neutrophil in the focus of future research on the possible roles of *P. gingivalis* in the development of autoimmunity in RA.

Supporting Information

Supplementary Table S1 - Neutrophil proteome data
Supplementary Table S2 - Macrophage proteome data
Figure S1 – Infection coverage in neutrophils and macrophages by PPAD-proficient or PPAD-deficient strains of *P. gingivalis*
Figure S2 – Strain-specific and PPAD-dependent impact of *P. gingivalis* on individual neutrophil proteins

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References

1. Cooper, G. S., Bynum, M. L. K. & Somers, E. C. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J. Autoimmun*. 33, 197–207 (2009).
2. Tonetti, M. S., Jepsen, S., Jin, L. & Otomo-Corgel, J. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. *J. Clin. Periodontol.* 44, 456–462 (2017).
3. de Smit, M. *et al.* Periodontitis in established rheumatoid arthritis patients: a cross-sectional clinical, microbiological and serological study. *Arthritis Res. Ther.* 14, R222 (2012).
4. van Winkelhoff, A. J., Loos, B. G., van der Reijden, W. A. & van der Velden, U. *Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. *J. Clin. Periodontol.* 29, 1023–8 (2002).
5. How, K. Y., Song, K. P. & Chan, K. G. *Porphyromonas gingivalis*: An Overview of Periodontopathic Pathogen below the Gum Line. *Front. Microbiol.* 7, 53 (2016).
6. van Winkelhoff, A. J., Appelmelk, B. J., Kippuw, N. & de Graaff, J. K-antigens in *Porphyromonas gingivalis* are associated with virulence. *Oral Microbiol. Immunol.* 8, 259–265 (1993).
7. Laine, M. L., Appelmelk, B. J. & van Winkelhoff, A. J. Prevalence and distribution of six capsular serotypes of *Porphyromonas gingivalis* in periodontitis patients. *J. Dent. Res* 76, 1840–1844 (1997).
8. Gabarrini, G. *et al.* The peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis*. *Sci. Rep.* **5,** 13936 (2015).
9. Gabarrini, G. *et al.* Conserved Citrullinating Exoenzymes in Porphyromonas Species. *J. Dent. Res* 97, 556–562 (2018).
10. Baka, Z. *et al.* Citrullination under physiological and pathological conditions. *Jt., Bone, Spine* 79, 431–6 (2012).
11. Montgomery, A. B. *et al.* Crystal structure of *Porphyromonas gingivalis* peptidylarginine deiminase: implications for autoimmunity in rheumatoid arthritis. *Ann. Rheum. Dis.* 75, 1255–1261 (2016).
12. Stobernack, T. *et al.* Extracellular Proteome and Citrullinome of the Oral Pathogen *Porphyromonas gingivalis*. *J. Proteome Res.* 15, 4532–4543 (2016).
13. Wegner, N. *et al.* Peptidylarginine deiminase from *Porphyromonas gingivalis* citrullinates human fibrinogen and α-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum.* 62, 2662–72 (2010).
14. Bielecka, E. *et al.* Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity. *J. Biol. Chem.* 289, 32481–32487 (2014).
15. Stobernack, T. *et al.* A Secreted Bacterial Peptidylarginine Deiminase Can Neutralize Human Innate Immune Defenses. *mBio* 9, 456 (2018).
16. Wegner, N. *et al.* Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. *Immunol. Rev.* 233, 34–54 (2010).
17. Cortés-Vieyra, R., Rosales, C. & Uribe-Querol, E. Neutrophil Functions in Periodontal Homeostasis. *J. Immunol. Res.* 2016, 1396106–9 (2016).
18. Park, M. H., Jeong, S. Y., Na, H. S. & Chung, J. *Porphyromonas gingivalis* induces autophagy in THP-1-derived macrophages. *Mol. Oral. Microbiol.* 32, 48–59 (2017).

19. Pathirana, R. D., O'Brien-Simpson, N. M., Visvanathan, K., Hamilton, J. A. & Reynolds, E. C. Flow cytometric analysis of adherence of *Porphyromonas gingivalis* to oral epithelial cells. *Infect. Immun*. 75, 2484–2492 (2007).

20. Igboin, C. O., Griffen, A. L. & Leys, E. J. *Porphyromonas gingivalis* strain diversity. *J. Clin. Microbiol.* 47, 3073–3081 (2009).

21. Rossi, A. & Lord, J. Adiponectin inhibits neutrophil phagocytosis of Escherichia coli by inhibition of PKB and ERK 1/2 MAPK signalling and Mac-1 activation. *PLoS ONE* 8, e69108 (2013).

22. Gabarrini, G. *et al.* There's no place like OM: Vesicular sorting and secretion of the peptidylarginine deiminase of *Porphyromonas gingivalis*. *Virulence* 9, 456–464 (2018).

23. Bonn, F. *et al.* Picking vanished proteins from the void: how to collect and ship/share extremely dilute proteins in a reproducible and highly efficient manner. *Anal. Chem.* 86, 7421–7 (2014).

24. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372 (2008).

25. Tyanova, S. *et al.* The Perseus computational platform for comprehensive analysis of (prote) omics data. *Nat. Methods* 13, 731–740 (2016).

26. Saeed, A. I. *et al.* TM4 microarray software suite. *Meth. Enzymol.* 411, 134–193 (2006).

27. Saeed, A. I. *et al.* TM4: a free, open-source system for microarray data management and analysis. *BioTechniques* 34, 374–378 (2003).

28. Lê, S., Josse, J. & Husson, F. FactoMineR: An R package for multivariate analysis. *J. Stat. Softw.* 25, 1–18 (2007).

29. Plotly Technologies Inc. *Collaborative data science Publisher: Plotly Technologies Inc.* (2015).

30. R Core Team. *R: A language and environment for statistical computing*. (2018).

31. Szklarczyk, D. *et al.* STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43, D447–52 (2015).

32. Neiders, M. E. *et al.* Heterogeneity of virulence among strains of Bacteroides gingivalis. 24, 192–198 (1989).

33. Miyata, J. *et al.* Cathepsin G: the significance in rheumatoid arthritis as a monocyte chemoattractant. *Rheumatol. Int.* 27, 375–382 (2007).

34. Matsumoto, T. *et al.* The membrane proteinase 3 expression on neutrophils was downregulated after treatment with infliximab in patients with rheumatoid arthritis. *Clin. Appl. Thromb. Hemost.* 14, 186–192 (2008).

35. Igboin, C. O., Moeschberger, M. L., Griffen, A. L. & Leys, E. J. *Porphyromonas gingivalis* virulence in a *Drosophila melanogaster* model. *Infect. Immun.* 79, 439–448 (2010).

36. Zheng, C., Wu, J. & Xie, H. Differential expression and adherence of *Porphyromonas gingivalis* FimA genotypes. *Mol. Oral. Microbiol.* 26, 388–395 (2011).

37. Hajishengallis, G., Wang, M., Harokopakis, E., Triantafilou, M. & Triantafilou, K. *Porphyromonas gingivalis* fimbriae proactively modulate beta2 integrin adhesive activity and promote binding to and internalization by macrophages. *Infect. Immun.* 74, 5658–5666 (2006).

38. Wang, M. & Hajishengallis, G. Lipid raft-dependent uptake, signalling and intracellular fate of *Porphyromonas gingivalis* in mouse macrophages. *Cell. Microbiol.* 10, 2029–2042 (2008).

39. Laine, M. L., Appelmelk, B. J. & van Winkelhoff, A. J. Novel polysaccharide capsular serotypes in *Porphyromonas gingivalis*. *J. Periodont. Res.* 31, 278–284 (1996).

40. Tarcsa, E. *et al.* Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. *J. Biol. Chem.* 271, 30709–30716 (1996).

41. Moelants, E. A. V. *et al.* Citrullination and proteolytic processing of chemokines by *Porphyromonas gingivalis*. *Infect. Immun.* 82, 2511–2519 (2014).

42. *Porphyromonas gingivalis* Manipulates Complement and TLR Signaling to Uncouple Bacterial Clearance from Inflammation and Promote Dysbiosis. *Cell Host Microbe* 15, 768–778 (2014).

43. Kammerl, I. E. & Meiners, S. Proteasome function shapes innate and adaptive immune responses. *Am. J. Physiol. Lung Cell Mol. Physiol.* 311, L328–36 (2016).

44. Nencioni, A., Grunebach, F., Patrone, F., Ballestrero, A. & Brossart, P. The proteasome and its inhibitors in immune regulation and immune disorders. *Crit. Rev. Immunol.* 26, 487–498 (2006).

45. Veiga, E. & Cossart, P. Ubiquitination of intracellular bacteria: a new bacteria-sensing system? *Trends Cell Biol.* 15, 2–5 (2005).

46. Iovino, F., Gradstedt, H. & Bijlsma, J. J. The Proteasome-Ubiquitin System Is Required for Efficient Killing of Intracellular *Streptococcus pneumoniae* by Brain Endothelial Cells. *mBio* 5, e00984–14–e00984–14 (2014).

47. Gabarrini, G. *et al.* Dropping anchor: attachment of peptidylarginine deiminase via A-LPS to secreted outer membrane vesicles of *Porphyromonas gingivalis*. *Sci. Rep.* 8, 8949 (2018).

CHAPTER 7

Summary and future perspectives

Summary

Hosting about 600 different bacterial species, the human mouth is one of the most diverse microbial habitats of the human body[1]. *Porphyromonas gingivalis* is one of these bacterial species. Although it constitutes less than 0.01% of the human oral microbiome, it is one of the key players in the highly prevalent oral diseases gingivitis and periodontitis [2]. *P. gingivalis* can shift the eubiotic healthy state of the mouth towards a dysbiotic state characterized by inflammation and destruction of the tissues surrounding the teeth. Recently, periodontitis and *P. gingivalis* have also been associated with the prominent inflammatory disease rheumatoid arthritis (RA), since individuals with periodontitis have a two times higher risk of developing RA than individuals with a healthy periodontium[3,4]. But how can a single low-abundant bacterial species contribute to such a massive shift in the oral microbiome, thereby impacting on the condition of the periodontium and various other sites of the human body, such as the synovial joints? As a first approach to tackle these questions, the aim of the PhD research documented in this thesis was to resolve the numerous roles of *P. gingivalis* in early-stage interactions with its human host. A special focus was placed on the production, secretion and delivery of virulence factors, with particular emphasis on the *P. gingivalis* peptidylarginine deiminase (PPAD).

Virulence factors are molecules (often proteins) that help a bacterium to colonize or invade different host tissues or body sites. To do so, they facilitate attachment, metabolic activity, host cell and tissue destruction and evasion of the host's immune defenses. There are numerous ways of studying these factors, which all follow the central principle of molecular biology, namely the transcription of DNA into RNA and translation of RNA into proteins. Accordingly, the present investigations were initiated with a study to assess whether the gene encoding PPAD belongs to the core genome of *P. gingivalis.* **Chapter 2** describes the strict conservation of this gene in a collection of 100 *P. gingivalis* isolates derived from Dutch periodontal clinics. Furthermore, database searches indicated that the PPAD enzyme is a unique feature of *P. gingivalis* among bacterial pathogens. Nonetheless, while the enzymatic activity of PPAD was known, the biological and clinical relevance of this enzyme was still poorly understood and debated. However, if PPAD were not important for the bacterium in its ecological niche, the oral cavity, the PPAD gene would probably not be part of the core genome. A second goal of the studies described in this chapter was to compare the citrullination capability of *P. gingivalis* isolates from periodontitis patients, RA patients and healthy control individuals. However, no clear difference in overall citrullination patterns could be detected for the different investigated isolates, which is consistent with the strict conservation of PPAD.

The next question to be addressed was whether those 100 clinical isolates actually express their PPAD genes. This was examined through the studies in **chapter 3**, where the subcellular localization of PPAD was also taken into account. From a bacterial perspective, it is of crucial importance that virulence factors are not only produced efficiently, but also secreted and delivered to an appropriate target location. To exert their effects on host cells, virulence factors have to be localized on the surface of the bacterial cell or in the extracellular milieu. In Gram-negative bacteria like *P. gingivalis,* this is achieved by several different mechanisms that facilitate the passage of virulence factors across the

inner and outer membranes of the cell envelope. Subsequent secretion may involve the release of the proteins in a water-soluble state or in association with outer-membrane vesicles (OMVs). In the case of PPAD, both ways of secretion are utilized as documented in chapter 3. In fact, most investigated isolates secrete PPAD mainly in the OMV-bound state and to a lesser extent as a soluble protein. However, some clinical isolates present a massive reduction of the OMV-bound version of PPAD, mainly secreting PPAD in a soluble form. This reduction in OMV-association of PPAD is genetically linked to an amino acid substitution where a lysine residue is present in position 373 instead of a glutamine residue. This substitution is of high interest from a molecular point of view, since it seems to directly inhibit the binding of PPAD to the outer membrane. However, this substitution cannot be associated with any clinical phenotype, suggesting that membrane-binding of PPAD per se is not crucial for interactions of *P. gingivalis* with the human host.

While chapter 2 and 3 solely focus on the role of PPAD as a virulence factor, **chapter 4** deals with the entire extracellular proteome and citrullinome of *P. gingivalis.* Citrullinated proteins have been implicated in the pathogenesis of RA, since patients with RA develop auto-antibodies against citrullinated residues up to 10 years before clinical signs of RA become apparent[5]. Aim of chapter 4 was, therefore, to explore which *P. gingivalis* proteins are efficiently secreted into the extracellular milieu and which of these proteins are citrullinated. By implementation of a state-of-the-art mass spectrometry (MS) approach, it was possible to detect around 250 extracellular proteins, of which six to 25 were found to be citrullinated. The citrullinated proteins included major virulence factors, like the protease RgpA and the adhesive fimbriae proteins. PPAD itself was also found to be citrullinated in some cases, which could be associated with a difference in post-translational modification. However, it should be noted here that the detection of protein citrullination is highly challenging and that in most available assays based on antibodies or particular reagents, small differences in the citrullination of individual arginine residues or even entire proteins may be overlooked. This also applies to the citrullination assay implemented for the studies in chapter 2. Therefore, the implementation of MS, as done for the studies documented in chapter 4 represented a major improvement. However, even with MS, the detection of citrullination remains challenging since the mass shift caused by citrullination is only 1 Dalton. Accordingly, a careful manual curation of the MS data is essential to show citrullination unambiguously. This was implemented in the present studies to show, for the first time, the bacterial proteins that are citrullinated and to uncover potential targets for future therapies against periodontitis and RA.

As mentioned above, the full spectrum of biological functions of PPAD was not known at the start of this PhD research. There were several previous studies that proposed PPAD as a virulence factor acting on numerous host cell types[6,7]. **Chapter 5** of this thesis documents for the first time which effects the PPAD enzyme has on the innate immune system. In particular, it was shown that PPAD can act in three distinct ways: *i.* inhibition of phagocytosis in neutrophils *ii.* impairment of neutrophil extracellular trap (NET) formation and *iii.* de-activation of the antimicrobial peptide LP9. All three mechanisms are crucial elements of the innate immune system and *P. gingivalis* can successfully escape these defenses via its potent PPAD enzyme. The importance of this enzyme was further underlined by the observation

that PPAD-deficient mutants of *P. gingivalis* were much less virulent in a *Galleria mellonella* infection model compared to the PPAD-proficient wild-type strains.

The research presented in the final experimental **chapter 6** was aimed at determining effects of *P. gingivalis* and PPAD on the proteome and citrullinome of human neutrophils and macrophages. In accordance with the observations described in chapter 5, PPAD is a critical factor in the down-regulation of host defense proteins, involved in phagocytosis, responses involving the formation of antimicrobial peptides and reactive oxygen species (ROS), and NET formation. Many of these important host defense factors were also found to be citrullinated. The present observations place the neutrophil in clear focus for future research on the roles of *P. gingivalis* in RA. This view is underscored by the unpublished observation that the growth of *P. gingivalis in vitro* can be enhanced significantly by providing human neutrophils as a 'nutritional supplement' (Fig. 1).

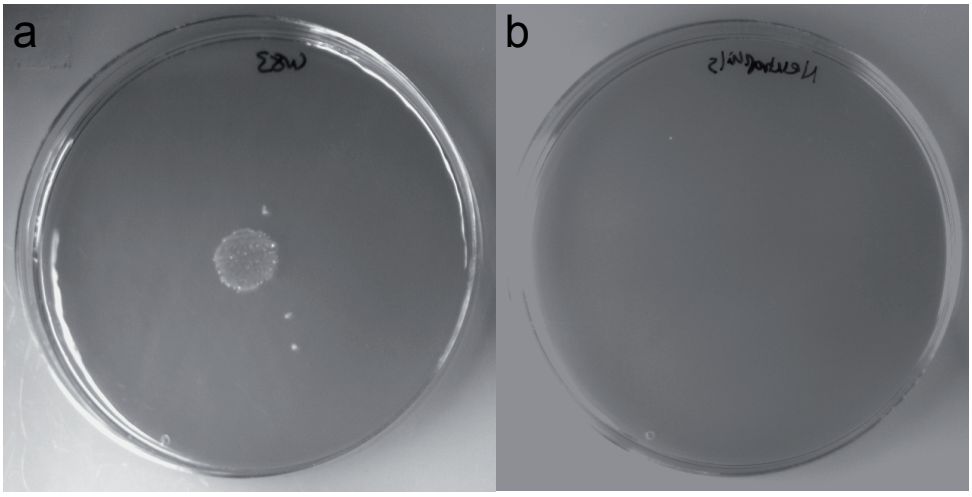

Figure 1: Neutrophils as a 'nutritional supplement' for *P. gingivalis* grown on Brain-Heart-Infusion agar plates. **(a)** *P. gingivalis* was streaked confluently on the surface of the plate and human neutrophils from a healthy donor were subsequently spotted in the center of the plate. **(b)** The same human neutrophil preparation as used in (a) was confluently plated as a control to verify the absence of bacterial contamination. Both plates were incubated for 5 days anaerobically at 37°C.

Future perspectives

A central question that remains to be answered is: what makes *P. gingivalis* a keystone periodontal pathogen? Besides the above-described PPAD enzyme, *P. gingivalis* possesses several other virulence factors accounting for its pathogenicity. These virulence factors are interesting research subjects for understanding the interactions of *P. gingivalis* with the human host, and they may eventually be exploited as novel targets for drug development. In this respect, proteases represent a particularly relevant class of virulence factors that may allow *P. gingivalis* to feed not only on gingival tissue, but even on the highly bactericidal human neutrophils (Fig. 1). *P. gingivalis* produces several proteases of different classes in large amounts, including trypsin-like, collagenolytic and glycylprolyl peptidases. Among the most prominent enzymes are the cysteine proteases, referred to as gingipains[8]. In fact, gingipains form the majority of proteases produced by *P. gingivalis*[9]. They are specific to *P. gingivalis*, responsible for the destruction of periodontal tissue and de-regulate host defense mechanisms, as observed in periodontitis[10].

The gingipains of *P. gingivalis* account for its trypsin-like activity[9]. Hitherto, two major types of gingipains are known, encoded by three genes. The arginine-specific gingipains RgpA and RgpB cleave polypeptide chains at arginine residues. The lysine-specific gingipain Kgp cleaves polypeptide chains at lysine residues[8]. The genes encoding these three gingipains are universally conserved in all *P. gingivalis* isolates[11]. Nevertheless, three types of *rgp* genes (type A, B and C) can be distinguished and two types of *kgp* genes (type I and II)[12].

The gingipain enzymes are known to exist in multiple forms. The molecular masses of major Rgps were for example shown to be 110-, 95-, 70-, 90-, and 50-kDa, which can be explained by their modular structure[9]. RgpA and Kgp form non-covalent complexes of a catalytic domain, bound to four polypeptides of hemagglutinin domains, whereas RgpB lacks a hemagglutinin domains (Fig. 2) [8]. Gingipains are found mostly bound to the outer membrane, but they are also secreted into the medium in an OMV-bound or soluble state[13]. An A-LPS membrane-anchor facilitates proper binding to the outer membrane upon secretion of the gingipains via the Porin secretion system (PorSS) [14]. The differences in molecular mass can be explained by the fact that the catalytic domains form complexes with multiple non-covalently bound hemagglutinin domains and a C-terminal domain (CTD) [9]. The catalytic domains of Kgp and RgpA are largely distinct, showing only 22% similarity. Their hemagglutinin regions are however very similar, though one region shows considerable variability. The catalytic domain of RgpB is nearly identical to that of RgpA[8,11]. However, there are notable differences in substrate specificity between the catalytic domains of RgpA and RgpB, which can be explained by four amino acids substitutions around the active site[15].

The RgpA and Kgp gingipains are produced as polyproteins, comprising the catalytic domains and the hemagglutinin/adhesion (HA) domains. These polyproteins need to be proteolytically processed to form mature, fully active enzymes. The HA domains are excised and subsequently stay non-covalently attached to the catalytic domains to form complexes. The lack of an adhesion binding motif in RgpB,

which is found in the catalytic domains of RgpA and Kgp, is thought to be the reason why RgpB is not capable of binding the HA domains[16].

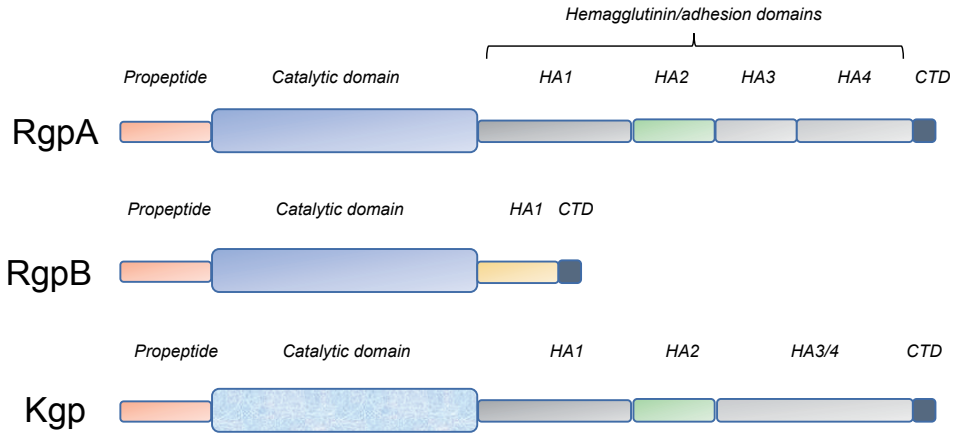

Figure 2: Polypeptide structure of the gingipains RgpA, RgpB and Kgp. The gingipains consist of a propeptide, a catalytic domain, hemagglutinin/adhesion (HA) domains and a C-terminal domain (CTD). Homology between the respective domains is indicated in color code. The size of the domains in the scheme is proportional to their molecular weight.

Processing of these polyproteins requires activity of the gingipains themselves and an additional carboxypeptidase[17]. RgpA and Kgp contain large N- and C-terminal extensions that require proteolytic processing at several arginine and lysine residues, and in the case of RgpB at arginine residues, to produce the mature enzymes[18]. Kgp needs to be processed by Rgps to activate its proteolytic activity[19]. Gingipains were furthermore implicated in the attachment of an A-lipopolysaccharide (A-LPS) anchor to the C-terminus[17]. In particular, RgpB appears to be involved in this post-translational modification, which also renders the enzymes more stable[20]. However, it was recently shown that the binding of PorSS substrates to the outer membrane is facilitated by a sortase-like enzyme, called PG0026, which substitutes the CTD with the A-LPS anchor[14].

Gingipains have been studied extensively and it is evident that they serve many crucial functions for the bacterium. This is exemplified by their requirement in nutrient- and iron acquisition, the involvement in tissue destruction, but also in biofilm formation and host colonization Moreover, the gingipains are involved in modulation of host immune system factors such as cytokines and receptors. In the following, several of these effects and functions will be addressed in more detail.

First of all, gingipains aid in providing nutrients for the bacterium. Since *P. gingivalis* is unable to utilize sugars as carbon and energy sources, this bacterium relies on peptides and amino acids provided through protein degradation by gingipains[21]. Gingipains are also key factors in iron acquisition by *P. gingivalis*. Free iron is insoluble in water at a physiological pH. For this reason, iron in biological systems

is generally only found in iron-binding complexes such as heme and hemoglobin. *P. gingivalis* can bind heme on its surface, which might provide a nutritional advantage and contributes to the formation of the black pigment. However, because of limited iron availability, the bacterium needs compensating mechanisms to obtain heme and iron in order to survive and proliferate. Remarkably, *P. gingivalis* does not use siderophores, small iron-binding molecules used by many other Gram-negative bacteria for iron uptake, but instead it employs various proteins for this purpose including the gingipains[8]. *P. gingivalis* can acquire heme from a range of hemoproteins, including hemoglobin and transferrin, which are also present in the gingival crevicular fluid in diseased periodontal pockets, indicating that the bacterium can capture heme from these proteins. Especially Kgp was found to be involved in this process[8]. RgpA and Kgp, but not RgpB, bind and degrade hemoglobin[22,23]. The fact that gingipains contain hemagglutinin domains, and are also involved in hemolysis, implies an involvement in vascular disruption, degradation of heme-binding proteins and subsequent utilization of the liberated iron by this pathogen[8]. For these reasons, gingipains were referred to as 'the molecular teeth of a microbial vampire'[16]. A recent study reported that the proteolytic activity of gingipains on hemoglobin is enhanced by small peptides such as glycylglycine (GlyGly) [24]. These peptides serve as acceptors for a gingipain transpeptidation activity that outcompetes their hydrolytic activity. Intriguingly, in the absence of GlyGly, gingipains were shown to produce up to 116 novel transpeptidation products of hemoglobin-derived peptides. Conceivably, such products could represent neo-epitopes that eventually lead to the breakdown of immunological tolerance of the host.

Besides providing essential nutrients, gingipains are also involved in survival through colonization and biofilm formation of *P. gingivalis*. To survive in the oral cavity, *P. gingivalis* must compete with other bacteria, but also form biofilm plaques with these co-resident species[12]. Of note, genetically-engineered bacteria deficient in gingipains showed no co-aggregation activity with *Actinomyces viscosus*, a commensal oral bacterium [25]. The same phenomenon was observed for co-aggregation with *Treponema denticola* and *Tannerella forsythia* (formerly known as *Bacteroides forsythus*), which are major pathogens in periodontal disease that are generally referred to as the 'red complex' [26,27].

A noteworthy feature of the gingipains is that they significantly contribute to the processing of various surface and secretory proteins. As mentioned above, gingipains perform self-cleavage, but they are also involved in the processing of other major virulence factors such as fimbriae, which are outer membrane structures that facilitate attachment to other bacteria and host cells. In particular, Rgps are involved in maturation and translocation of the precursors of fimbrilin, which is a major constituent of fimbriae. An Rgp mutant, but not a Kgp mutant, indeed showed less fimbriation compared to the respective wild-type strains[28].

Many studies provide evidence that gingipains are involved in tissue destruction. For this purpose, their adhesion domains are not only involved in colonization and interaction with other bacteria, but also in binding human epithelial cells and tissues[29]**.** Thus, RgpA and Kgp bind to fibrinogen, laminin, collagen type V, fibronectin and hemoglobin. This implies that the adhesion domains target the proteolytic activity to host cell (matrix) proteins. The continuous binding and degradation of surface

proteins on host cells can lead to cell death, inflammation, tissue destruction and vascular disruption. In turn, the latter can cause gingival bleeding, which is a major symptom of periodontitis[16].

Not only direct disruption of endothelial cells causes vascular disruption and bleeding. Gingipains can also increase vascular permeability indirectly via the kinin system. Higher production of proinflammatory kinins has been suggested to contribute to periodontitis progression, and RgpA and Kgp appear to have the ability of kinin activation[30]. Gingipain-dependent release of vasoactive kinins might contribute to vascular permeability and increase gingival edema and crevicular fluid production in periodontitis[31]. Furthermore, endothelial adhesion molecule expression is affected by gingipain activity, resulting in reduced leukocyte adhesion and recruitment to the site of inflammation[32]. Regarding the role of *P. gingivalis* in periodontal tissue destruction, the group of gingipains appear to be a major factor[33,34] that does not only degrade adhesion molecules, but also the structural proteins collagen type I and type IV [10].

A characteristic feature of periodontitis is alveolar bone resorption by osteoclasts. Gingipains may also be stimulators in this process. In particular, Kgp may enhance osteoclastogenesis by the degradation of osteoprotegerin, an osteoclastogenesis inhibitory factor secreted by osteoblasts[35]. The degradation of osteoprotegerin is suggested to be a crucial event in osteoclastogenesis and bone loss in periodontitis. Conversely, a recent study showed that differentiation into osteoclasts induced by the pro-inflammatory cytokine IL-17A can be reduced by Kgp degradation[36].

Besides the above-mentioned processes that lead to tissue destruction, gingipains are also involved in degradation of immune system components thereby facilitating the evasion of host defense mechanisms and colonization. Strikingly, Rgps are not affected by natural human protease inhibitors such as cystatins and alpha 1-antichymotrypsin, and also Kgp was shown to retain its activity in human plasma, suggesting that gingipains are 'immune' to normal host defense systems[10,37]. Gingipains even have the ability to cleave immunoglobulin G, and its cleavage products were detected in gingival crevicular fluid samples from severe periodontitis patients[37]. Not only immunoglobulins are degraded, also complement factors and receptors are cleaved and degraded[38,39]. It has been shown that Kgp significantly hinders opsonin-dependent phagocytosis of *P. gingivalis* by neutrophils[37]. Additionally, gingipains are involved in activation of the coagulation system and platelet aggregation via activation of the coagulation factors human factor IX and thrombin. Therefore, gingipains may be involved in subsequent production of prostaglandins and interleukin 1, all associated with the development of periodontitis. Possibly this explains the presence of bacterial components in atherosclerosis plaques and thrombus sites, all indicating a relationship between periodontitis and cardiovascular diseases[40,41].

Not only immune factors, but also immune cells are influenced by the multifunctional gingipains. In macrophages, RgpA and Kgp decrease expression of the innate immune receptor CD14. Reduced CD14 correlates with decreased TNFα production and bacterial phagocytosis. As a consequence, macrophages appear to become hypo-responsive to bacterial challenge[42]. Even when *P. gingivalis* is phagocytosed, it can evade the autophagic pathway in infected cells and traffic to the endocytic pathway. In this respect, gingipains are required to gain resistance to lysosomal destruction[43].

Gingipains are able to degrade cytokines and chemoattractant proteins produced by human cells[44]. It has been proposed that gingipains efficiently degrade pro-inflammatory cytokines and receptors close to the infection site while, more distant from the infection site, lower gingipain levels can cause enhanced inflammatory responses through protease-activated receptors (PAR) activation and cytokine release, ultimately leading to tissue destruction and bone resorption[16]. Gingival epithelial cells use PARs to recognize *P. gingivalis,* but the expression of certain PARs changes after exposure to the supernatant of cultured *P. gingivalis*[45].

Besides macrophages, also neutrophils seem to be impaired by the *P. gingivalis* gingipains. The bactericidal function of neutrophils is inhibited by gingipains[46]. The production of ROS from activated neutrophils is also inhibited by Rgps, suggesting that they are responsible for disruption of the function of these cells[10]. The neutrophils are extensively involved in the inflammatory response to *P. gingivalis* infection, and the modulation of their behavior by gingipains is highly relevant in view of the fact that neutrophils play a major role in the immune response against *P. gingivalis*[47].

Judged by their multiple targets, it must be concluded that gingipains are major virulence factors of *P. gingivalis* and that their protease activity has an impressive variety of direct and indirect effects on the interaction with the human host. For these reasons, it is important to investigate their exact function and role in the onset and progression of the disease, as well as their potential for the development of vaccines and novel antimicrobial therapies. Recently, it was shown that immunization with a recombinant RgpA protein protects mice against periodontitis induced by *P. gingivalis* and decreases alveolar bone loss by 50% [48] . Curtis *et al.* demonstrated that Kgp might be the most promising drug target amongst the gingipains. A specific slowly reversible inhibitor was developed for Kgp. This inhibitor causes loss of pigmentation, poor growth and significant reduction of virulence in mice[49]. The latter observations underpin the important role of Kgp in nutrient acquisition and virulence of *P. gingivalis.* Therefore, inhibitors of these proteases might form a novel class of antimicrobial agents. Accordingly, recombinant derivatives of RgpA and Kgp have been administered as a vaccine to assess the ability of different sub-domains of these proteins to attenuate *P. gingivalis* infection in mice. From this experiment it was concluded that all adhesion domains significantly attenuated the infection, but the first adhesion domain had the highest efficacy. This domain contains sub-domains implicated in host tissue binding. Importantly, the resulting antisera reacted with Kgp, RgpA and HagA[29]. The efficacy of this vaccine might be due to the fact that it targets several *P. gingivalis* proteins simultaneously. Nevertheless, in an earlier study, the adhesion domains demonstrated high immunogenicity, but a peptide derivative thereof did not elicit a protective immune response. A major conclusion from this study was that vaccination against the N-terminus of the catalytic domain of Rgps is protective, which might be due to inactivation of the enzyme by antibody recognition of a processing site on the precursor[50]. Even though different studies propose the use of different gingipain epitopes for immunization, it is clear that the gingipains can provide good targets for novel therapeutic strategies. Therefore, it is of great importance to investigate the role of these proteases in the survival and virulence of *P. gingivalis* and the interaction with immune cells in the human host.

In recent years, a plethora of clinical isolates of *P. gingivalis* and related *Porphyromonas* species has been characterized by whole genome sequencing[51,52]. Many of these genome sequences are publicly available, which prompted an investigation into the genetic conservation of gingipains in *Porphyromonas* species isolates from humans and non-human hosts. Figure 3 illustrates the genetic conservation of the three gingipains in these isolates. It is evident that the N-terminal parts of the gingipains, including the catalytic domains, are highly conserved in isolates from humans. In contrast, there is substantial variation in the sequences encoding the HA domains. This variation is even more prominent when gingipain gene sequences from human-derived *Porphyromonas* isolates are compared with those of isolates derived from non-human hosts. The heterogeneity in the HA domain sequences places the catalytic domain in focus as the main region to be targeted in future vaccines. Of note, vaccines directed against the HA domains might protect against a subset of *P. gingivalis* isolates, but definitely not against all of them.

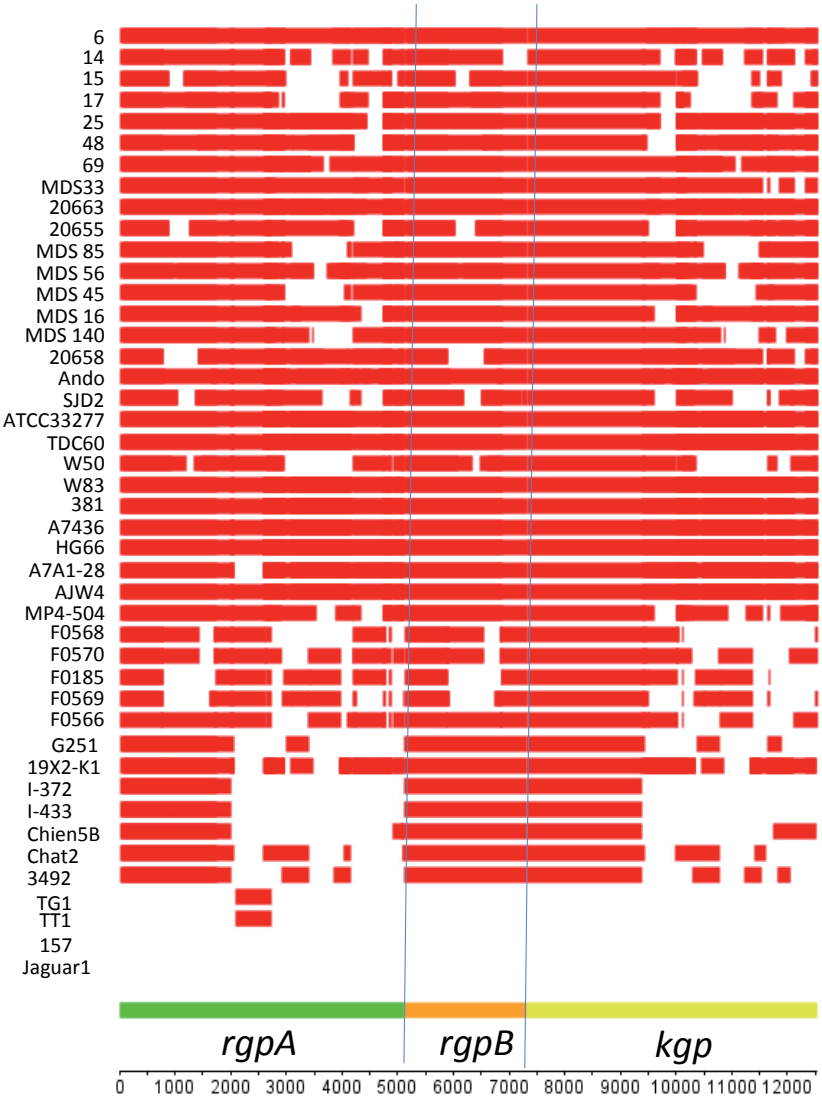

Figure 3: Genetic variation of the gingipains RgpA, RgpB and Kgp. DNAplotter analysis of the genetic variation of the three gingipain genes *rgpA, rgpB* and *kgp* using the respective genes of ATCC 32277 as a reference. Red regions represent highly conserved sequences in gingipain genes from different isolates compared to the respective reference genes, while white regions indicate high sequence divergence. The first 33 isolates in the alignment are human-derived *P. gingivalis,* while the last 11 isolates are *Porphyromonas* isolates derived from non-human hosts. 19X2-K1, *P. gingivalis* isolate derived from a monkey; G251, I-372, I-433, Chien 5B, Chat 2 and 3492 are *Porphyromonas gulae* isolates derived from monkeys, a dog and cats; TG 1 and TT1 are *Porphyromonas loveana* isolates from sheep; 157 is a *Porphyrpomonas salivosa* isolate from a cat; and Jaguar 1 is a *Porphyropmonas circumdentaria* isolate from a jaguar[52].

Since *P. gingivalis* possesses a number of very potent virulence factors, like PPAD and the gingipains, it is able to shift the delicate homeostasis in the oral environment towards a dysbiotic condition. However, as mentioned before, *P. gingivalis* only constitutes a very small proportion of the whole oral microbiome[2]. Future research should therefore not only focus on the role of single bacterial species, but should also investigate the complex interactions of different species of bacteria in the oral cavity. One possible approach could be the co-culturing of the red complex bacteria *P. gingivalis*, *T. denticola* and *T. forsythia* with a subsequent analysis of the secretion of important virulence factors. This is an informative approach, as was previously demonstrated for a number of wound-colonizing bacteria that were shown to modulate each other's expression of virulence factors[53]. Alternative approaches would be the implementation of shotgun metagenome or metatranscriptome sequencing to determine the abundance of millions of microbial genes and their expression in individuals with periodontitis, healthy individuals or individuals with RA. Thereby it would become possible to have a deep look into the whole oral microbiome, also including viruses and fungi. However, the latter approaches would not provide any information on the actual presence of the microbial proteins in the mouth, but only on the respective genes and transcripts. Therefore, it would be important to complement such studies by metaproteomic studies. However, before metatranscriptomic and metaproteomic approaches can be implemented, it will be important to develop biochemical and bioinformatic approaches to effectively separate human and bacterial transcripts and proteins.

Besides RA, there are a few other autoimmune diseases which have been recently associated with microbial colonization or infection. Bacteria and other microbes closely interact with the human immune system, as described for *P. gingivalis* in the present thesis. Therefore, it is conceivable that these microbes tip the balance from a healthy immune status towards a dysfunctional, hyperactive or autoimmune state. For examples, this has been proposed for the autoimmune disease granulomatosis with polyangiitis (GPA), where nasal carriage of the infectious agent *Staphylococcus aureus* has been associated with a higher risk of relapses in these patients[54,55]. Another interesting example is provided by the gut bacterium *Enterococcus gallinarum,* which can travel towards other tissues like the lymph nodes, the liver or the spleen. There it can trigger the onset of autoimmune diseases like systemic lupus and autoimmune liver disease[56]. Intriguingly, researchers were able to suppress autoimmunity in mice with an antibiotic or a vaccine directed against *E. gallinarum*.

At this point, the precise mechanisms how bacteria can trigger autoimmunity in RA, GPA or systemic lupus are unknown. In view of the severe impact of autoimmune diseases on the patients' quality of life, future research should clearly investigate how single bacterial species or altered microbiomes can lead to such severe systemic effects. Most probably, with this future knowledge, it should become possible to prevent or treat chronic inflammatory diseases in patients by a therapy as simple as a vaccination against 'bad' bacteria or a supplementation of beneficial bacteria via the administration of probiotics. This would be a major advance towards healthy ageing and increased quality of life for many people who suffer from chronic diseases.

References

1. Dewhirst, F. E. *et al.* The human oral microbiome. *J. Bacteriol.* 192, 5002–5017 (2010).
2. Hajishengallis, G., Darveau, R. P. & Curtis, M. A. The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* 10, 717–725 (2012).
3. Detert, J., Pischon, N., Burmester, G. R. & Buttgereit, F. The association between rheumatoid arthritis and periodontal disease. *Arthritis Res. Ther.* 12, 218 (2010).
4. de Smit, M. J., Brouwer, E., Vissink, A. & van Winkelhoff, A. J. Rheumatoid arthritis and periodontitis; a possible link via citrullination. *Anaerobe* 17, 196–200 (2011).
5. Toes, R. E. & van der Woude, D. ACPA (anti-citrullinated protein antibodies) and rheumatoid arthritis. *Acta Reumatol. Port.* 36, 205–207 (2011).
6. Bielecka, E. *et al.* Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity. *J. Biol. Chem.* 289, 32481–32487 (2014).
7. Maresz, K. J. *et al. Porphyromonas gingivalis* facilitates the development and progression of destructive arthritis through its unique bacterial peptidylarginine deiminase (PAD). *PLoS Pathog.* 9, e1003627 (2013).
8. Olczak, T., Simpson, W., Liu, X. & Genco, C. A. Iron and heme utilization in *Porphyromonas gingivalis*. *FEMS Microbiol. Rev.* 29, 119–144 (2005).
9. Potempa, J., Pike, R. & Travis, J. The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-gingipain or Lys-gingipain. *Infect. Immun.* 63, 1176–1182 (1995).
10. Kadowaki, T., Yoneda, M., Okamoto, K., Maeda, K. & Yamamoto, K. Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*. *J. Biol. Chem.* 269, 21371–21378 (1994).
11. Mikolajczyk-Pawlinska, J. *et al.* Genetic variation of *Porphyromonas gingivalis* genes encoding gingipains, cysteine proteinases with arginine or lysine specificity. *Biol. Chem.* 379, 205–211 (1998).
12. Tribble, G. D., Kerr, J. E. & Wang, B.-Y. Genetic diversity in the oral pathogen *Porphyromonas gingivalis*: molecular mechanisms and biological consequences. *Future Microbiol.* 8, 607–620 (2013).
13. Seers, C. A. *et al.* The RgpB C-terminal domain has a role in attachment of RgpB to the outer membrane and belongs to a novel C-terminal-domain family found in *Porphyromonas gingivalis*. *J. Bacteriol.* 188, 6376–6386 (2006).
14. Gorasia, D. G. *et al. Porphyromonas gingivalis* Type IX Secretion Substrates Are Cleaved and Modified by a Sortase-Like Mechanism. *PLoS Pathog.* 11, e1005152 (2015).
15. Ally, N. *et al.* Characterization of the specificity of arginine-specific gingipains from *Porphyromonas gingivalis* reveals active site differences between different forms of the enzymes. *Biochemistry* 42, 11693–11700 (2003).
16. O-Brien-Simpson, N. M., Veith, P. D., Dashper, S. G. & Reynolds, E. C. *Porphyromonas gingivalis* gingipains: the molecular teeth of a microbial vampire. *Curr. Protein Pept. Sci.* 4, 409–426 (2003).
17. Potempa, J., Sroka, A., Imamura, T. & Travis, J. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: structure, function and assembly of multidomain protein complexes. *Curr. Protein Pept. Sci.* 4, 397–407 (2003).

18. Aduse-Opoku, J. *et al.* Generation of lys-gingipain protease activity in *Porphyromonas gingivalis* W50 is independent of Arg-gingipain protease activities. *Microbiology (Reading, Engl.)* 146 (Pt 8), 1933–1940 (2000).

19. Okamoto, K., Kadowaki, T., Nakayama, K. & Yamamoto, K. Cloning and sequencing of the gene encoding a novel lysine-specific cysteine proteinase (Lys-gingipain) in *Porphyromonas gingivalis*: structural relationship with the arginine-specific cysteine proteinase (Arg-gingipain). *J. Biochem.* 120, 398–406 (1996).

20. Rangarajan, M. *et al.* Expression of Arg-Gingipain RgpB is required for correct glycosylation and stability of monomeric Arg-gingipain RgpA from *Porphyromonas gingivalis* W50. *Infect. Immun.* 73, 4864–4878 (2005).

21. Kadowaki, T. *et al. Porphyromonas gingivalis* proteinases as virulence determinants in progression of periodontal diseases. *J. Biochem.* 128, 153–159 (2000).

22. Simpson, W., Olczak, T. & Genco, C. A. Lysine-specific gingipain K and heme/hemoglobin receptor HmuR are involved in heme utilization in *Porphyromonas gingivalis*. *Acta Biochim. Pol.* 51, 253–262 (2004).

23. Olczak, T., Dixon, D. W. & Genco, C. A. Binding specificity of the *Porphyromonas gingivalis* heme and hemoglobin receptor HmuR, gingipain K, and gingipain R1 for heme, porphyrins, and metalloporphyrins. *J. Bacteriol.* 183, 5599–5608 (2001).

24. Zhang, L. *et al. Porphyromonas gingivalis* Gingipains Display Transpeptidation Activity. *J. Proteome Res.* 17, 2803–2818 (2018).

25. Abe, N. *et al.* Roles of Arg- and Lys-gingipains in coaggregation of *Porphyromonas gingivalis*: identification of its responsible molecules in translation products of rgpA, kgp, and hagA genes. *Biol. Chem.* 385, 1041–1047 (2004).

26. Ito, R., Ishihara, K., Shoji, M., Nakayama, K. & Okuda, K. Hemagglutinin/Adhesin domains of *Porphyromonas gingivalis* play key roles in coaggregation with Treponema denticola. *FEMS Immunol. Med. Microbiol.* 60, 251–260 (2010).

27. Yoneda, M. *et al.* Mixed infection of *Porphyromonas gingivalis* and *Bacteroides forsythus* in a murine abscess model: involvement of gingipains in a synergistic effect. *J. Periodont. Res.* 36, 237–243 (2001).

28. Kadowaki, T. *et al.* Arg-gingipain acts as a major processing enzyme for various cell surface proteins in *Porphyromonas gingivalis*. *J. Biol. Chem.* 273, 29072–29076 (1998).

29. Frazer, L. T. *et al.* Vaccination with recombinant adhesins from the RgpA-Kgp proteinase-adhesin complex protects against *Porphyromonas gingivalis* infection. *Vaccine* 24, 6542–6554 (2006).

30. Rapala-Kozik, M. *et al.* Adsorption of components of the plasma kinin-forming system on the surface of *Porphyromonas gingivalis* involves gingipains as the major docking platforms. *Infect. Immun.* 79, 797–805 (2011).

31. Monteiro, A. C. *et al.* Kinin danger signals proteolytically released by gingipain induce Fimbriae-specific IFN-gamma- and IL-17-producing T cells in mice infected intramucosally with *Porphyromonas gingivalis*. *J. Immunol.* 183, 3700–3711 (2009).

32. Yun, P. L. W., Decarlo, A. A. & Hunter, N. Gingipains of *Porphyromonas gingivalis* modulate leukocyte adhesion molecule expression induced in human endothelial cells by ligation of CD99. *Infect. Immun.* 74, 1661–1672 (2006).

33. Baba, A. *et al.* Arg-gingipain is responsible for the degradation of cell adhesion molecules of human

gingival fibroblasts and their death induced by *Porphyromonas gingivalis*. *Biol. Chem.* 382, 817–824 (2001).

34. Sheets, S. M., Potempa, J., Travis, J., Fletcher, H. M. & Casiano, C. A. Gingipains from *Porphyromonas gingivalis* W83 synergistically disrupt endothelial cell adhesion and can induce caspase-independent apoptosis. *Infect. Immun.* 74, 5667–5678 (2006).

35. Yasuhara, R. *et al.* Lysine-specific gingipain promotes lipopolysaccharide- and active-vitamin D3-induced osteoclast differentiation by degrading osteoprotegerin. *Biochem. J.* 419, 159–166 (2009).

36. Akiyama, T. *et al. Porphyromonas gingivalis*-derived lysine gingipain enhances osteoclast differentiation induced by tumor necrosis factor-α and interleukin-1β but suppresses that by interleukin-17A: importance of proteolytic degradation of osteoprotegerin by lysine gingipain. *J. Biol. Chem.* 289, 15621–15630 (2014).

37. Vincents, B. *et al.* Cleavage of IgG1 and IgG3 by gingipain K from *Porphyromonas gingivalis* may compromise host defense in progressive periodontitis. *FASEB J.* 25, 3741–3750 (2011).

38. Jagels, M. A. *et al.* Cleavage of the human C5A receptor by proteinases derived from *Porphyromonas gingivalis*: cleavage of leukocyte C5a receptor. *Adv. Exp. Med. Biol.* 389, 155–164 (1996).

39. Jagels, M. A., Travis, J., Potempa, J., Pike, R. & Hugli, T. E. Proteolytic inactivation of the leukocyte C5a receptor by proteinases derived from *Porphyromonas gingivalis*. *Infect. Immun.* 64, 1984–1991 (1996).

40. Imamura, T., Tanase, S., Hamamoto, T., Potempa, J. & Travis, J. Activation of blood coagulation factor IX by gingipains R, arginine-specific cysteine proteinases from *Porphyromonas gingivalis*. *Biochem. J.* 353, 325–331 (2001).

41. Imamura, T., Banbula, A., Pereira, P. J., Travis, J. & Potempa, J. Activation of human prothrombin by arginine-specific cysteine proteinases (Gingipains R) from *Porphyromonas gingivalis*. *J. Biol. Chem.* 276, 18984–18991 (2001).

42. Wilensky, A., Tzach-Nahman, R., Potempa, J., Shapira, L. & Nussbaum, G. *Porphyromonas gingivalis* gingipains selectively reduce CD14 expression, leading to macrophage hyporesponsiveness to bacterial infection. *J. Innate Immun.* 7, 127–135 (2015).

43. Kadowaki, T. *et al.* A role for gingipains in cellular responses and bacterial survival in *Porphyromonas gingivalis*-infected cells. *Front. Biosci.* 12, 4800–4809 (2007).

44. Baba, A., Kadowaki, T., Asao, T. & Yamamoto, K. Roles for Arg- and Lys-gingipains in the disruption of cytokine responses and loss of viability of human endothelial cells by *Porphyromonas gingivalis* infection. *Biol. Chem.* 383, 1223–1230 (2002).

45. Zhang, D., Li, S., Hu, L., Sheng, L. & Chen, L. Modulation of protease-activated receptor expression by *Porphyromonas gingivalis* in human gingival epithelial cells. *BMC Oral Health* 15, 128 (2015).

46. Nakayama, K., Kadowaki, T., Okamoto, K. & Yamamoto, K. Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas gingivalis*. Evidence for significant contribution of Arg-gingipain to virulence. *J. Biol. Chem.* 270, 23619–23626 (1995).

47. Wingrove, J. A. *et al.* Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas* (Bacteroides) gingivalis. *J. Biol. Chem.* 267, 18902–18907 (1992).

48. Wilensky, A., Potempa, J., Houri-Haddad, Y. & Shapira, L. Vaccination with recombinant

RgpA peptide protects against *Porphyromonas gingivalis*-induced bone loss. *J. Periodont. Res.* 52, 285–291 (2017).

49. Curtis, M. A. *et al.* Attenuation of the virulence of *Porphyromonas gingivalis* by using a specific synthetic Kgp protease inhibitor. *Infect. Immun.* 70, 6968–6975 (2002).

50. Genco, C. A., Potempa, J., Mikolajczyk-Pawlinska, J. & Travis, J. Role of gingipains R in the pathogenesis of *Porphyromonas gingivalis*-mediated periodontal disease. *Clin. Infect. Dis.* 28, 456–465 (1999).

51. Gabarrini, G. *et al.* Dropping anchor: attachment of peptidylarginine deiminase via A-LPS to secreted outer membrane vesicles of *Porphyromonas gingivalis*. *Sci. Rep.* 8, 8949 (2018).

52. Gabarrini, G. *et al.* Conserved Citrullinating Exoenzymes in *Porphyromonas* Species. *J. Dent. Res* 97, 556–562 (2018).

53. García-Pérez, A. N. *et al.* From the wound to the bench: exoproteome interplay between wound-colonizing *Staphylococcus aureus* strains and co-existing bacteria. *Virulence* 9, 363–378 (2018).

54. Popa, E. R., Stegeman, C. A., Kallenberg, C. G. M. & Tervaert, J. W. C. *Staphylococcus aureus* and Wegener's granulomatosis. *Arthritis Res.* 4, 77–79 (2002).

55. Glasner, C. *et al.* Low anti-staphylococcal IgG responses in granulomatosis with polyangiitis patients despite long-term *Staphylococcus aureus* exposure. *Sci. Rep.* 5, 8188 (2015).

56. Manfredo Vieira, S. *et al.* Translocation of a gut pathobiont drives autoimmunity in mice and humans. *Science* 359, 1156–1161 (2018).

CHAPTER 8

Nederlandse samenvatting
List of publications
Biography
Acknowledgements – Dankwort – Dankwoord

Nederlandse samenvatting

Met meer dan 600 verschillende soorten bacteriën en andere micro-organismen is de mond één van de meest diverse microbiële habitats in het menselijk lichaam[1]. *Porphyromonas gingivalis* is één van deze bacteriesoorten. Hoewel *P. gingivalis* minder dan 0,01% van ons orale microbioom vertegenwoordigt, is deze bacterie één van de belangrijkste spelers bij ziektes in de mond, zoals de veel voorkomende tandvleesontstekingen en parodontitis[2]. *P. gingivalis* is in staat om het microbiële evenwicht in een gezonde mond naar een ongezonde "dysbiotische" situatie te laten kantelen, die gekenmerkt wordt door ontsteking en destructie van de weefsels die de tanden omgeven, het zogenaamde parodontium. Onlangs zijn parodontitis en *P. gingivalis* ook in verband gebracht met de ernstige ontstekingsziekte reumatoïde artritis (RA), omdat personen met parodontitis een twee keer hoger risico hebben om RA te ontwikkelen dan personen met een gezond parodontium[3,4]. Maar hoe kan een enkele, in lage hoeveelheden voorkomende bacteriesoort bijdragen aan grote veranderingen in het orale microbioom en daardoor een impact hebben op de toestand van het parodontium en diverse andere delen van het menselijk lichaam, zoals de synoviale gewrichten? Het doel van het promotieonderzoek beschreven in dit proefschrift was de vele rollen van *P. gingivalis* in de interacties met zijn humane gastheer te onderzoeken. Speciale nadruk werd daarbij gelegd op de productie van gesecreteerde virulentiefactoren en het *P. gingivalis* peptidyl-arginine-deïminase (PPAD) in het bijzonder. Dit enzym katalyseert de omzetting van positief geladen arginine residuen in een eiwitketen tot neutrale citrulline residuen in een proces dat eiwitcitrullinering genoemd wordt. Het gevolg hiervan is dat het gecitrullineerde eiwit verandert met betrekking tot zijn lading, vorm, activiteit en herkenbaarheid door het humane immuunsysteem.

Virulentiefactoren zijn moleculen (vaak eiwitten) die een bacterie helpen om verschillende gastheerweefsels of lichaamsdelen te koloniseren of binnen te dringen. Hiertoe faciliteren ze de hechting van de bacterie aan gastheercellen en weefsels, het verkrijgen van nutriënten ten koste van de gastheer, weefseldestructie en eliminatie of ontwijking van het immuunsysteem. Er zijn verschillende manieren om virulentiefactoren te bestuderen die allemaal de basisprincipes van de moleculaire biologie volgen, namelijk de transcriptie van DNA in RNA en de translatie van RNA in eiwitten. Dienovereenkomstig is het hier beschreven onderzoek begonnen met de vraag of het gen, dat PPAD codeert, behoort tot het kerngenoom van *P. gingivalis.* In **Hoofdstuk 2** van dit proefschrift wordt aangetoond dat dit gen strikt geconserveerd is in een 100-tal klinische *P. gingivalis* isolaten die verkregen werden uit patiënten met parodontitis. Verder werd door middel van database-onderzoek aangetoond, dat het PPAD-enzym een uniek kenmerk is van *P. gingivalis*, dat in geen enkele andere humane ziekteverwekker voorkomt. Terwijl de enzymatische activiteit van PPAD reeds bekend was bij aanvang van het hier beschreven onderzoek, was de biologische en klinische relevantie van dit enzym op dat moment nog niet duidelijk. Als PPAD echter niet belangrijk zou zijn voor de *P. gingivalis* bacterie in de ecologische niche van de mond, dan zou het PPAD-gen waarschijnlijk geen deel uitmaken van het kerngenoom. Daarom was een tweede doel van de studies, beschreven in hoofdstuk 2, om het citrullinerende vermogen van *P. gingivalis* isolaten uit parodontitis patiënten,

RA-patiënten en gezonde individuen te vergelijken. Er kon echter geen duidelijk verschil in de algemene citrullineringspatronen gedetecteerd worden bij de verschillende onderzochte isolaten, wat consistent is met de strikte conservering van het PPAD-gen.

Een volgende vraag die beantwoord moest worden was of de 100 klinische *P. gingivalis* isolaten hun PPAD-genen daadwerkelijk tot expressie brengen. Dit werd onderzocht met behulp van de experimenten beschreven in **hoofdstuk 3,** waarbij de sub-cellulaire lokalisatie van PPAD geanalyseerd werd. Vanuit een bacterieel perspectief is het van cruciaal belang dat virulentiefactoren niet alleen efficiënt geproduceerd worden, maar ook dat ze worden uitgescheiden en afgeleverd op een geschikte doellocatie in de gastheer. Om hun effecten op gastheercellen effectief uit te oefenen, moeten virulentiefactoren daarom op het oppervlak van de bacteriële cel of in het extracellulaire milieu gelokaliseerd zijn. In Gram-negatieve bacteriën, zoals *P. gingivalis*, wordt dit doel in eerste instantie bereikt met behulp van verschillende eiwittransportsystemen die de passage van virulentiefactoren vanuit het cytoplasma over de binnen- en buitenmembranen van de celenvelop faciliteren. De daaropvolgende secretie van virulentiefactoren in het gastheermilieu kan op twee manieren plaatsvinden, namelijk in de vorm van individuele wateroplosbare eiwitten of in associatie met membraanblaasjes die van de buitenmembraan afgesnoerd worden. Laatstgenoemde membraanblaasjes worden daarom vaak “outer membrane vesicles” of OMVs genoemd. Voor PPAD worden beide manieren van secretie simultaan door de *P. gingivalis* bacterie gebruikt, zoals beschreven in hoofdstuk 3. De meeste onderzochte isolaten scheiden PPAD voornamelijk in de OMV-gebonden toestand uit en in mindere mate als een oplosbaar eiwit. Een beperkt aantal klinische isolaten scheidt PPAD echter voornamelijk in een oplosbare vorm uit. De reductie van PPAD-associatie met OMVs is genetisch geassocieerd met een specifieke aminozuursubstitutie, waarbij een glutamine-residu op positie 373 in de eiwitketen vervangen is door een lysine-residu. Deze substitutie lijkt de binding van PPAD aan de buitenmembraan in sterke mate te remmen. Desondanks kan de waargenomen aminozuursubstitutie niet geassocieerd worden met een bepaald ziektebeeld, hetgeen suggereert dat de OMV-associatie van PPAD niet per sé essentieel is voor de interactie van *P. gingivalis* met de menselijke gastheer.

Terwijl de hoofdstukken 2 en 3 van dit proefschrift zich met name richten op de rol van PPAD als een gesecreteerde virulentiefactor, handelt **hoofdstuk 4** over het volledige extracellulaire proteoom en citrullinoom van *P. gingivalis.* Gecitrullineerde eiwitten zijn betrokken bij de pathogenese van RA, aangezien patiënten met RA in verhoogde mate auto-antistoffen tegen gecitrullineerde eiwitten ontwikkelen. Dit proces begint vaak al 10 jaar voordat de klinische symptomen van RA zich manifesteren[5]. Doel van het onderzoek beschreven in hoofdstuk 4 was daarom om te bepalen welke *P. gingivalis*-eiwitten efficiënt worden uitgescheiden in het extracellulaire milieu en welke van deze eiwitten door PPAD gecitrullineerd zijn. Door de implementatie van geavanceerde massaspectrometrie (MS) was het mogelijk om ongeveer 250 extracellulaire eiwitten te detecteren waarvan, afhankelijk van het geanalyseerde isolaat, zes tot 25 eiwitten gecitrullineerd bleken te zijn. De gecitrullineerde eiwitten omvatten belangrijke virulentiefactoren, zoals de protease RgpA en de fimbriae waarmee *P. gingivalis* zich aan humane weefsels kan hechten. PPAD bleek in sommige gevallen ook zelf gecitrullineerd te zijn. Bij deze studie moet opgemerkt worden, dat de ondubbelzinnige detectie van eiwitcitrullinatie

technisch zeer uitdagend is door het kleine massaverschil van slechts 1 Dalton, dat gepaard gaat met deze eiwitmodificatie. Hierdoor kunnen met name kleine variaties in eiwitcitrullinatie of zelfs volledige gecitrullineerde eiwitten gemakkelijk over het hoofd gezien worden. Dit laatste geldt overigens in nog sterkere mate voor de citrullinatie-assay die gebruikt werd voor de studies in hoofdstuk 2. Daarom vormde de implementatie van MS voor de detectie van eiwitcitrullinatie, zoals beschreven in hoofdstuk 4, een aanzienlijke technologische verbetering, waarmee voor het eerst aangetoond kon worden welke bacteriële eiwitten daadwerkelijk gecitrullineerd worden. Deze eiwitten vormen potentiële aangrijpingspunten voor toekomstige therapieën tegen parodontitis en RA, met name gezien de ogenschijnlijk belangrijke rol van gecitrullineerde eiwitten bij het ontstaan van deze aandoeningen.

Zoals hierboven vermeld, was het volledige spectrum van biologische functies van PPAD nog niet bekend bij de aanvang van dit promotieonderzoek ondanks verschillende eerdere studies, waarin PPAD voor het eerst gepresenteerd werd als een mogelijke virulentiefactor die aangrijpt op verschillende gastheercellen[6,7]. **Hoofdstuk 5** van dit proefschrift documenteert voor het eerst welke effecten het PPAD-enzym heeft op het aangeboren immuunsysteem dat belangrijke bacterie-killers omvat, waaronder immuuncellen zoals neutrofielen en positief-geladen antimicrobiële peptides. Het bleek dat PPAD op drie verschillende manieren kan werken, namelijk door *i.* de remming van opname en destructie van *P. gingivalis* door neutrofielen, *ii.* inhibitie van de vorming van zogenaamde “neutrophil extracellular traps” (NETs) bestaande uit DNA-structuren, waarin *P. gingivalis* letterlijk door neutrofielen gevangen kan worden, en *iii.* de neutralisatie van positief-geladen antimicrobiële peptides zoals het LP9-peptide, dat afgesplitst wordt van het humane antibacteriële eiwit lysozym. Alle drie mechanismen zijn cruciale elementen van het aangeboren immuunsysteem en *P. gingivalis* kan klaarblijkelijk succesvol aan deze afweermechanismen ontsnappen met behulp van het gesecreteerde PPAD-enzym. Het belang van PPAD werd verder onderstreept door de observatie, dat PPAD-deficiënte mutanten van *P. gingivalis* veel minder virulent waren in een dierlijk infectiemodel, de wasmot *Galleria mellonella*, vergeleken met de PPAD-producerende wild-type stammen.

Het onderzoek gepresenteerd in het laatste experimentele **hoofdstuk 6** was gericht op het bepalen van de effecten van *P. gingivalis* en PPAD op het proteoom en citrullinoom van menselijke immuuncellen, te weten de neutrofielen en macrofagen. In overeenstemming met de waarnemingen beschreven in hoofdstuk 5 blijkt PPAD in deze studies ook een kritische factor in de regulatie van afweereiwitten van de gastheer die betrokken zijn bij opname en destructie van bacteriën en de vorming van NETs. Meerdere van deze belangrijke afweerfactoren van de gastheer blijken ook gecitrullineerd te worden. Alle resultaten tezamen beschouwd lijkt het er op dat met name de interacties tussen *P. gingivalis* en neutrofielen een hoofdrol spelen bij parodontitis en RA. Deze interacties verdienen daarom extra aandacht bij toekomstig onderzoek naar de exacte rol die *P. gingivalis* speelt bij het ontstaan van deze ziekte. Deze mening wordt nog eens onderstreept door de ongepubliceerde waarneming, dat de groei van *P. gingivalis in vitro* aanzienlijk gestimuleerd kan worden door humane neutrofielen aan te bieden als een soort ‘voedingssupplement’ voor de bacterie (**hoofdstuk 7**).

Samenvattend kan geconcludeerd worden dat het huidige promotieonderzoek een aantal belangrijke nieuwe inzichten heeft opgeleverd in de vroege interacties tussen *P. gingivalis* en het

humane immuunsysteem, waarbij het PPAD-enzym een sleutelrol vervult. Op dit moment zijn de precieze mechanismen die vervolgens bepalen hoe *P. gingivalis* de auto-immuniteit zou kunnen veroorzaken die leidt tot RA nog grotendeels onbekend. Met het oog op de ernstige consequenties van auto-immuunziekten zoals RA voor de kwaliteit van leven van patiënten met deze ziekte zou toekomstig onderzoek zich vooral moeten richten op de vraag, hoe een enkele bacterie de samenstelling van het mond-microbioom kan ontregelen en de ernstige systemische effecten kan veroorzaken die met RA gepaard gaan. Met name onderzoek naar de onderliggende causale verbanden en de directe of indirecte rol van PPAD verdient nader onderzoek. Wellicht zal het dan met de verworven kennis mogelijk zijn om chronische ontstekingsziekten als RA te voorkomen of te behandelen door een therapie die zo eenvoudig is als een vaccinatie tegen 'slechte' bacteriën of een toediening van nuttige bacteriën in de vorm van probiotica. Dergelijke therapieën zouden een belangrijke stap voorwaarts zijn in het streven om gezond ouder te worden en de kwaliteit van leven voor veel mensen die aan chronische ziektes lijden te verhogen.

Referenties

1. Dewhirst, F. E. *et al.* The human oral microbiome. *J. Bacteriol.* 192**,** 5002–5017 (2010).
2. Hajishengallis, G., Darveau, R. P. & Curtis, M. A. The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* 10, 717–725 (2012).
3. Detert, J., Pischon, N., Burmester, G. R. & Buttgereit, F. The association between rheumatoid arthritis and periodontal disease. *Arthritis Res. Ther.* 12, 218 (2010).
4. de Smit, M. J., Brouwer, E., Vissink, A. & van Winkelhoff, A. J. Rheumatoid arthritis and periodontitis; a possible link via citrullination. *Anaerobe* 17, 196–200 (2011).
5. Toes, R. E. & van der Woude, D. ACPA (anti-citrullinated protein antibodies) and rheumatoid arthritis. *Acta Reumatol. Por*t. 36, 205–207 (2011).
6. Bielecka, E. *et al.* Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity. *J. Biol. Chem.* 289, 32481–32487 (2014).
7. Maresz, K. J. *et al. Porphyromonas gingivalis* facilitates the development and progression of destructive arthritis through its unique bacterial peptidylarginine deiminase (PAD). *PLoS Pathog.* 9, e1003627 (2013).

List of publications

de Vries SPW, **Stobernack T**, Pereira RR, Pelsser LM, ter Braak CJF, Aarts E, van Baarlen P, Kleerebezem M, Frankena K, Hontelez S. **Biomarker Research in ADHD: the Impact of Nutrition (BRAIN). An open-label trial to investigate the mechanisms underlying the effects of a few-foods diet on ADHD symptoms in children.** *Submitted*

Stobernack T, Palma Medina LM, du Teil Espina M, Piebenga DR, Otto A, Sura T, Becher D, de Jong A, van Winkelhoff AJ, Brouwer E, Westra J, Heeringa P, van Dijl JM. ***Porphyromonas gingivalis*** **and its secreted peptidylarginine deiminase modulate the proteome of human neutrophils and macrophages.** *Submitted*

Zhao X, Palma Medina LM, **Stobernack T**, Glasner C, de Jong A, Utra P, Setroikromo R, Quax WJ, Otto A, Becher D, Buist G, van Dijl JM. **Exoproteome heterogeneity among closely related *Staphylococcus aureus* t437 isolates and possible implications for virulence.** *Journal of Proteome Research. 2019*

Stobernack T, du Teil Espina M, Mulder LM, Palma Medina LM, Piebenga DR, Gabarrini G, Zhao X, Janssen KMJ, Hulzebos J, Brouwer E, Sura T, Becher D, van Winkelhoff AJ, Götz F, Otto A, Westra J, van Dijl JM. **A Secreted Bacterial Peptidylarginine Deiminase Can Neutralize Human Innate Immune Defenses.** *mBio. 2018 Oct 30;9(5). pii:e01704-18*

Nepal S, Bonn F, Grasso S, **Stobernack T**, de Jong A, Zhou K, Wedema R, Rosema S, Becher D, Otto A, Rossen JW, van Dijl JM, Bathoorn E. **An ancient family of mobile genomic islands introducing cephalosporinase and carbapenemase genes in *Enterobacteriaceae*.** *Virulence. 2018;9(1):1377-1389*

Gabarrini, G, Palma Medina LM, **Stobernack T**, Prins RC, du Teil Espina M, Kuipers J, Chlebowicz MA, Rossen JWA, van Winkelhoff AJ, van Dijl JM. **There's no place like OM: Vesicular sorting and secretion of the peptidylarginine deiminase of *Porphyromonas gingivalis*.** *Virulence. 2018 Jan 1;9(1):456-464*

Stobernack T, Glasner C, Junker S, Gabarrini G, de Smit M, de Jong A, Otto A, Becher D, van Winkelhoff AJ, van Dijl JM. **Extracellular Proteome and Citrullinome of the Oral Pathogen *Porphyromonas gingivalis*.** *Journal of Proteome Research 2016 Dec 2;15(12):4532-4543*

Gabarrini G, de Smit M, Westra J, Brouwer E, Vissink A, Zhou K, Rossen JW, **Stobernack T**, van Dijl JM, van Winkelhoff AJ. **The peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis*.** *Scientific Reports 2015 Sep 25;5:13936*

Glasner C, van Timmeren MM, **Stobernack T**, Omansen TF, Raangs EC, Rossen JW, de Goffau MC, Arends JP, Kampinga GA, Koedijk DG, Neef J, Buist G, Tavakol M, van Wamel WJ, Rutgers A, Stegeman CA, Kallenberg CG, Heeringa P, van Dijl JM. **Low anti-staphylococcal IgG responses in granulomatosis with polyangiitis patients despite long-term *Staphylococcus aureus* exposure.** *Scientific Reports. 2015 Feb 2;5:8188*

van der Kooi-Pol MM, Sadaghian Sadabad M, Duipmans JC, Sabat AJ, **Stobernack T**, Omansen TF, Westerhout-Pluister GN, Jonkman MF, Harmsen HJ, van Dijl JM. **Topography of distinct *Staphylococcus aureus* types in chronic wounds of patients with epidermolysis bullosa.** *PLoS One. 2013 Jun 25;8(6):e67272*

Biography

Tim Stobernack was born on the 23rd of September 1989 in Wesel, Germany. After graduating at the Konrad-Duden-Gymnasium in Wesel in 2009, he studied 'Biological Sciences' at the University of Münster, Germany. In the last year of this study, he specialized in Biomedical Sciences and investigated several biochemical pathways involved in blood coagulation. He received his Bachelor of Science degree with a *cum laude* distinction in 2012. From 2012 to 2014, he followed the Topmaster program 'Medical and Pharmaceutical Drug Innovation' at the University of Groningen in the Netherlands, where he initiated the work on *Porphyromonas gingivalis* in the van Dijl lab during his first internship. He spent his second internship in the Weyand lab at Stanford University in the USA, investigating the metabolism of T cells in rheumatoid arthritis patients. He received his Master of Science degree with a *cum laude* distinction in 2014. At the end of his Master studies, he wrote a PhD research proposal under the supervision of Prof. van Dijl on the interaction of *P. gingivalis* with the human immune system, for which he received a fellowship from the Graduate School of Medical Sciences of the University of Groningen. In September 2014, he started his PhD research at the Department of Medical Microbiology of the University Medical Center Groningen, which resulted in the present thesis. Since September 2018, he is employed as a postdoctoral researcher in the chair group Host-Microbe Interactomics at the Department of Animal Sciences of the University of Wageningen, the Netherlands.

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Here it is, the final part of my thesis, in which I would like to thank all the people that supported and helped me in any way during the long journey of obtaining my PhD. I can say right from the start, that I am not a great novel writer, so I hope that you do not expect any professional novel writing or an acknowledgements section of 20 pages. I will try to be concise and to the point and still mention everyone in a way they deserve to be thanked.

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Tim
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